In re Application of: Lior GEPSTEIN et al      Examiner: Anoop Kumar Singh
Serial No.: 10/759,734      Group Art Unit: 1632
Filed: January 20, 2004      Attorney Docket: 27395
Office Action Mailing Date: January 6, 2009

## REMARKS

Reconsideration of the above-identified application in view of the amendments above and the remarks following is respectfully requested.

Claims 1-177, 182-186 and 196-199 are in this Application. Claims 1-175 and 182-185 have been withdrawn from consideration. Claims 176-177, 186 and 196-199 have been rejected. New claim 200 has now been added.

### *35 U.S.C. § 102 Rejections*

The Examiner has rejected claims 176-177, 186 and 196-199 under 35 U.S.C. § 102(e) as being anticipated by Funk et al. (US 6667176).

The Examiner states that Funk et al. teach an isolated a 4 day in-vitro suspension of EBs which is transferred to polyornithine-coated plates for additional 7 days to obtain beating cells exhibiting a cardiomyocyte phenotype.

The Examiner states that the claimed invention is a product and not a product by process and that Applicant's arguments as to how the product is obtained are not pertinent to the claimed product. The Examiner further states that if the prior art teaches a chemical structure identical to the claimed invention, the properties the Applicant discloses and/or claims are necessary present in the prior art.

The Examiner appears to have overlooked the clear differences which exist between the methodology of the prior art and the effect of the culturing methodology of the present on the structure and properties of the product formed.

The approach presented by the present inventors to generation and isolation of cardiomyocytes is radically different and a departure from the teachings of the prior art. Whereas, the prior art has taught (for over a decade prior to filing of the instant application) that cardiac lineage cells can only be derived from EBs which are cultured to the cystic stage, the present inventors have uncovered that unexpectedly, non-cystic EBs are a better source of cardiomyocytes provided that such EBs are cultured under conditions which prevent further differentiation into the cystic phenotype.

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Applicant disagrees with the Examiner's assessment that Funk et al. teach isolated human embryoid bodies which <u>include non-cystic embryoid bodies composed of cells having a cardiac phenotype</u> simply because the culturing times reported by Funk et al. although capable of producing cardiomyocyte progenitors are incapable of producing <u>non-cystic embryoid bodies</u> which include such progenitors.

In Example 5 of US 6667176, Funk et al. teaches the following:

*"The hES cells were dissociated into small clumps ..., and cultured in suspension to form embryoid bodies (EBs) ... <u>After 4 days</u> in suspension, the aggregates were transferred onto polyornithine-coated plates, <u>and cultured for additional 7 days</u>. The cultures were then examined for the presence of beating cells, and processed for immunohistochemistry."* (emphasis added)

Thus, Funk et al. teaches a culturing period of 4 days in suspension and 7 days on poly-ornithine plates. As a result the EBs of Funk et al. were subjected to a differentiation period of <u>11 days</u> since as is clearly stated by Funk et al., poly-ornithine coated plates induce, rather than suspend differentiation (Column 17, lines 9-16):

*"Briefly, a suspension of undifferentiated pPS cells is prepared, and then plated <u>onto a solid surface that promotes differentiation</u>. Suitable substrates include glass or plastic surfaces that are adherent, for example, <u>by coating with a polycationic substance, such as a polyamine or polyornithine</u>"* (emphasis added)

These time periods of culturing and appearance of cardiomyocyte progenitors correspond well to the time periods taught by the prior art in generating <u>cystic EBs</u> with beating cell clusters.

It is well known that "Human EBs frequently progress through a series of stages beginning as simple, morula-like EBs <u>eventually</u> forming <u>cavitated and cystic</u>

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EBs between 7 and 14 days of post-differentiation development" (page 195, column 2 of Odorico et al., STEM CELLS 2001;19:193-204, enclosed herewith).

Since Funk et al. differentiates the EBs for 11 days it is clear that the resultant EB culture generated thereby will not include non-cystic EBs.

Indeed, Funk et al. cite the teachings of O'shea [Anat. Rec. - New Anat. 257:323, 1999, article enclosed herewith) as the only reference for methodology suitable for generating EBs which include cardiomyocyte progenitors.

In context of producing such EBs O'shea teaches that:

"The presence of foci of spontaneously contracting muscle fibers after 8–20 days in vitro is one of the most striking aspects of EB differentiation. These cultures are typically initiated in suspension, and then resulting embryoid bodies are plated onto adhesive substrates to achieve final differentiation." (page 38, right column under "Muscle").

The EBs of the present invention were generated by culturing human embryonic stem cells for 7-10 days under non-adherent conditions and then transferring the formed EBs to gelatin-coated plates which froze the formed EBs in a non-differentiated, non-cystic morphological state (as opposed to the culture of Funk et al. which was plated under differentiating conditions and thus the plated EBs will continue to differentiate to form the cystic phenotype).

Thus, the culturing approach of the present invention results in a vastly different EB culture both morphologically (non-vacuated) and in terms of the quantity and quality of cardiomyocyte progenitors included therein.

Notwithstanding from the above, Applicant has elected to add New claim 200 which describes a culture which consists essentially of non-cystic EBs with cardiomyocyte progenitors.

The Examiner has also rejected claims 176, 177, 186 and 196-199 under 35 USC 102(e) as being anticipated by Thomson et al. (US 7220584 - effective filing date Feb 21, 2000).

The Examiner states that Thomson et al. teach an in vitro culture of human EBs containing cells that differentiate to cardiac phenotype.  The Examiner further states that Thomson teaches culturing of 11 days in suspension followed by mechanical or chemical dispersal and reattachment of dispersed cells to gelatin coated tissue culture plates.

Thomson et al propose further differentiation of EBs in order to obtain cells of cardiac lineage (column 5, lines 26-38):

*"Embryoid bodies can be differentiated into a variety of desired lineages."*(emphasis added)

*"If cardiac lineages are desired one could use techniques analogous to T. Doetschman et al., 87 J. Embry. Exper. Morph. 27 45 (1985). One could plate the bodies in serum-containing medium with no additives."*

Doetschman et al. (article enclosed herewith) observed that:

*"After approximately 11 days of culture, many cystic structures were present (Fig. 3C)"*

and that cardiac lineage cells appear in 30 % of cystic EBs (see abstract and Page 42 bottom).

*"About one third of the cystic embryoid bodies produced by the blastocyst-derived cells develop rhythmically contracting, intercalated disk-containing myocardial cells. The associated endocardial tissue found in cultures of substrate-attached cells can also form in the cystic structures (not shown). These data show that ES cells have the potential to develop into several cardiac cell types in a well-organized manner, suggesting that they may be suitable for investigations of heart organogenesis."*

Thus, Doetschman et al. teach that cardiac lineage cells appear in EBs that have been cultured to the cystic morphological state.

Thomson et al. follow the teachings of Doetschman et al. and culture EBs for a time period sufficient for generation of the cystic phenotype.  Following such culturing,

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Thomson et al continue to culture the EBs under differentiating conditions in order to obtain cystic EBs with cardiac lineage cells as taught by Doetschman et al.

Thus, contrary to the Examiners Assertion, Thompson et al. do not teach non-vacuated EBs that include cardiomyocyte progenitors but rather follow previously published guidelines for producing cystic EBs which include cardiac lineage cells.

It is therefore the Applicant's strong opinion that the teachings of Thomson et al. do not anticipate or render obvious (e.g. in combination with Carpenter and Igelmund) the present invention as claimed.

In view of the above amendments and remarks it is respectfully submitted that claims 176, 177, 186 and 196-200 are now in condition for allowance. A prompt notice of allowance is respectfully and earnestly solicited.

Respectfully submitted,

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Registration No. 40,338

Date: May 11, 2009

**_Enclosures:_**

☐ Petition for Extension (Two Months)
☐ Request for Continued Examination (RCE)
☐ References: Odorico et al.
            O'shea
            Doetschman et al.

# STEM CELLS

## Concise Review

# Multilineage Differentiation from Human Embryonic Stem Cell Lines

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## ABSTRACT

Stem cells are unique cell populations with the ability
to undergo both self-renewal and differentiation. A wide
variety of adult mammalian tissues harbors stem cells, yet
"adult" stem cells may be capable of developing into only
a limited number of cell types. In contrast, embryonic stem
(ES) cells, derived from blastocyst-stage early mammalian
embryos, have the ability to form any fully differentiated
cell of the body. Human ES cells have a normal karyotype,
maintain high telomerase activity, and exhibit remark-
able long-term proliferative potential, providing the pos-
sibility for unlimited expansion in culture. Furthermore,
they can differentiate into derivatives of all three embry-
onic germ layers when transferred to an in vivo environ-
ment. Data are now emerging that demonstrate human

ES cells can initiate lineage-specific differentiation pro-
grams of many tissue and cell types in vitro. Based on
this property, it is likely that human ES cells will provide
a useful differentiation culture system to study the mech-
anisms underlying many facets of human development.
Because they have the dual ability to proliferate indefi-
nitely and differentiate into multiple tissue types, human
ES cells could potentially provide an unlimited supply of
tissue for human transplantation. Though human ES
cell-based transplantation therapy holds great promise to
successfully treat a variety of diseases (e.g., Parkinson's
disease, diabetes, and heart failure) many barriers
remain in the way of successful clinical trials. Stem Cells
2001;19:193-204

## INTRODUCTION

Stem cells have the ability to choose between prolonged
self-renewal and differentiation. This fate choice is highly reg-
ulated by intrinsic signals and the external microenvironment,
the elements of which are being rapidly elucidated [1]. Stem
cells can be identified in many adult mammalian tissues. In
some tissues, such as epithelia, blood, and germline, stem cells
contribute to replenishment of cells lost through normal cellu-
lar senescence or injury. Stem cells may also be present in
other adult organs, such as the brain and pancreas, which nor-
mally undergo very limited cellular regeneration or turnover.

Although stem cells in adult tissues may have more "plas-
ticity" than originally thought, they typically form only a lim-
ited number of cell types. Stem cells of the early mammalian
embryo, in contrast, have the potential to form any cell type.
In the unmanipulated blastocyst-stage embryo, stem cells of
the inner cell mass (ICM) promptly differentiate to generate
primitive ectoderm, which ultimately differentiates during
gastrulation into the three embryonic germ (EG) layers. When
removed from their normal embryonic environment and cul-
tured under appropriate conditions, ICM cells give rise to
cells that proliferate and replace themselves indefinitely. Yet,

while in this undifferentiated state in culture, they maintain the developmental potential to form advanced derivatives of all three EG layers [2, 3]. ICM cells are the source cells from which pluripotent mouse, nonhuman primate, and human embryonic stem (ES) cells are generally derived, although there is evidence that mouse ES cells may be more closely related to primitive ectoderm [4-9].

The objective of this review is to describe the derivation and unique properties of human ES cells. Particular emphasis will be given to summarizing recent studies that focus on the potential of human ES cells for multilineage differentiation in vitro and in vivo. We will also outline key scientific questions that will need to be answered before the full therapeutic potential of human ES cells can be realized.
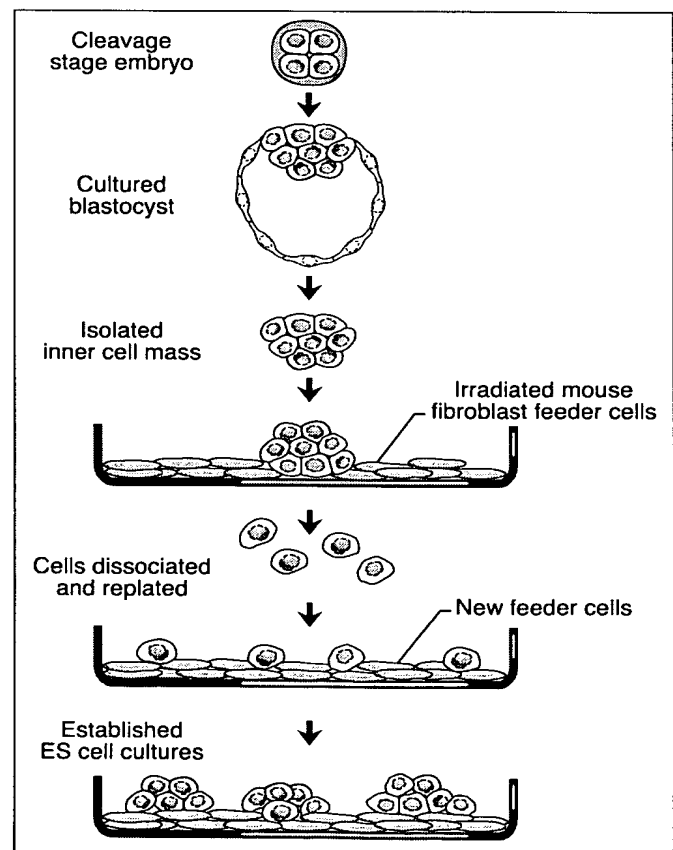
## DERIVATION OF HUMAN ES CELLS

The origin of human ES cells from the pre-implantation embryo is the defining feature that distinguishes ES cell lines from other pluripotent human cell lines, namely human embryonal carcinoma (EC) cell and human EG cell lines [7, 8, 10, 11]. EC cell lines are pluripotent cell lines derived from the undifferentiated stem cell components of spontaneously arising germ cell tumors found occasionally in certain strains of mice and humans [12, 13]. Pluripotent EG cell lines have been derived from mouse and human primordial germ cells from the genital ridges of fetuses [11, 14]. Years before the isolation of human EG or ES cells, EC cell lines from both mouse and later human teratocarcinomas provided an important in vitro model of differentiation [15].

Human ES cells have been derived from the ICM of blastocyst-stage embryos in essentially the same manner as rhesus monkey ES cells [6, 7]. Cleavage-stage human embryos, produced by in vitro fertilization for clinical purposes, are donated by individuals after informed consent. After embryos are grown to the blastocyst stage, the ICM is isolated and plated onto mitotically inactivated murine embryonic fibroblast (MEF) feeder layers in tissue culture (Fig. 1). The ICM cell outgrowths are propagated in the presence of serum, and colonies with the appropriate undifferentiated morphology are subsequently selected and expanded. After the initial derivation in serum, human ES cell lines can be maintained and propagated on feeder layers in medium containing serum alone or serum replacement medium and basic fibroblast growth factor (bFGF). Initially, human ES and EG cell lines were not clonally derived and so pluripotency could only be demonstrated for a population of cells. As such, the possibility existed that within a colony there were subpopulations of cells already committed to different lineages and no individual cell was capable of differentiating into derivatives of all three EG layers. Subsequently, clonally derived human ES cell lines, H9.1 and H9.2, were produced that retain all the

properties of the parental ES cell line, including the ability to generate teratomas in vivo harboring derivatives of all three EG layers [16].

## PROPERTIES OF HUMAN ES CELLS

Human and nonhuman primate ES cells share a similar morphology that is distinct from human EG cells and mouse ES cells [4, 6, 7, 11]. Human ES cells form relatively flat, compact colonies that easily dissociate into single cells in trypsin or in $Ca^{+2}$- and $Mg^{+2}$-free medium, whereas human EG cells form tight, more spherical colonies that are refractory to standard dissociation methods, but which more closely resemble the morphology of mouse ES cell colonies. Moreover, human ES cells grow more slowly than mouse ES cells; the population-doubling time of mouse ES cells is ~12 hours, whereas the population-doubling time of human ES cells is about 36 hours [16].



*Figure 1. Derivation of human ES cell lines. Human blastocysts were grown from cleavage-stage embryos produced by in vitro fertilization. ICM cells were separated from trophectoderm by immunosurgery, plated onto a fibroblast feeder substratum in medium containing fetal calf serum. Colonies were sequentially expanded and cloned.*

Paralleling these differences in cellular morphology, human ES cells differ from their murine counterparts with regard to cell-surface antigen phenotype. Like undifferentiated primate ES cells and human EC cells, human ES cells express stage-specific embryonic antigens 3 and 4 (SSEA-3 and SSEA-4), high molecular weight glycoproteins TRA-1-60 and TRA-1-81, and alkaline phosphatase [7, 17]. Undifferentiated mouse ES cells do not express SSEA-3 or SSEA-4, but do express the lactoseries glycolipid SSEA-1, which is not expressed in human ES cells, rhesus ES cells, or human EC cells [7, 17]. The functional significance of these antigens is unknown.

Human ES cells also differ from mouse ES cells in their in vitro culture requirements for undifferentiated growth. Mouse ES cells require leukemia inhibitory factor (LIF) for undifferentiated proliferation. In contrast, LIF alone is not sufficient to prevent differentiation of human ES cells in vitro [7, 18]. Instead, continued undifferentiated propagation of human ES cells currently require feeder layers and either the presence of serum or, if cultured in serum-free medium, bFGF [7, 16]. Under conditions of low cell density, human ES cell lines are more difficult to propagate in serum, with a cloning efficiency of approximately 0.25% [16]. In contrast, culture in both serum replacement medium and supplemental bFGF significantly increases the cloning efficiency over culture in serum alone [16]. Fibroblast feeder layers are currently required to prevent differentiation of human ES cells. How undifferentiated proliferation can be sustained in the absence of feeder cells is an area of active investigation. The critical factors produced by fibroblast feeder layers, which prevent differentiation of human ES cells, are entirely unknown. Further work is clearly needed to clarify the mechanisms involved in sustaining human ES cell proliferation, including specific receptor-ligand interactions, downstream signaling events, and cellular target molecules. Ultimately, it would be essential to establish feeder-independent culture conditions, which permit large-scale propagation of human ES cells in culture.

Human ES cells have demonstrated remarkably stable karyotypes. Human ES cell lines demonstrate normal XX and XY karyotypes, similar to ES cell lines from other species, but distinct from human EC lines derived from teratocarcinomas [7]. This characteristic makes human ES cells more relevant as a model for the study of developmental biology mechanisms and for derivation of differentiated cells for transplantation therapy.

Human ES cells express high levels of telomerase. The expression of telomerase, a ribonucleoprotein that adds telomere repeats to chromosome ends, thereby maintaining their length, is highly correlated with immortality in human cell lines [19]. Most diploid somatic cells do not express high levels of telomerase and enter replicative senescence after a finite proliferative life span in tissue culture, usually after 50-80 population doublings. Unique among normal somatic cells, some populations of adult stem cells (i.e., hematopoietic stem cells) in vivo also constituitively express telomerase [20, 21]; however, telomerase activity is not sustained when cells are placed in culture. In contrast, cells of the early embryo have high telomerase activity levels [22, 23]. Likewise, human ES cell lines exhibit high telomerase activity levels even after more than 300 population doublings and passage for more than 1 year in culture [7, 16]. In summary, properties of cells of the early embryo, such as normal karyotype and high telomerase activity, are sustained for an extended period of time by human ES cell lines in culture. This unique property among human cell lines has important implications as a tool to study cellular senescence and mechanisms of stem cell renewal.

## MULTILINEAGE DIFFERENTIATION IN VITRO

When removed from feeder layers and transferred to suspension culture, ES cells begin to differentiate into multicellular aggregates of differentiated and undifferentiated cells, termed embryoid bodies (EBs), which resemble early postimplantation embryos. Human EBs frequently progress through a series of stages beginning as simple, morula-like EBs eventually forming cavitated and cystic EBs between 7 and 14 days of post-differentiation development (Figs. 2A, B) [24]. As for mouse and nonhuman primate ES cells, differentiation in vitro is consistently disorganized and frequently variable from one EB to another within the same culture. A more comprehensive understanding of the morphology of human EBs and the relationships among different cell types comprising these complex embryo-like structures may yield important new information on early inductive events in human development.

Human ES cells, like nonhuman primate ES cells, are able to differentiate into trophoblast in culture. Nonhuman primate ES cell lines spontaneously differentiate in vitro into extraembryonic endoderm lineages, including yolk sac, and into trophoblast, as evidenced by α-fetoprotein and chorionic gonadotropin (CG) mRNA synthesis, and bioactive CG production [6]. Similarly, human EBs synthesize α-fetoprotein transcripts and secrete α-fetoprotein and hCG into the culture medium [7]. Human ES cells, therefore, represent a useful model in which to study human placental development and function.

Mouse ES cell lines are able to differentiate in vitro into a variety of embryonic and adult cell types from all three EG layers. These include cardiomyocytes, hematopoietic progenitors, yolk sac, skeletal myocytes, smooth muscle cells, adipocytes, chondrocytes, endothelial cells, melanocytes, neurons, glia, pancreatic islet cells, and primitive endoderm
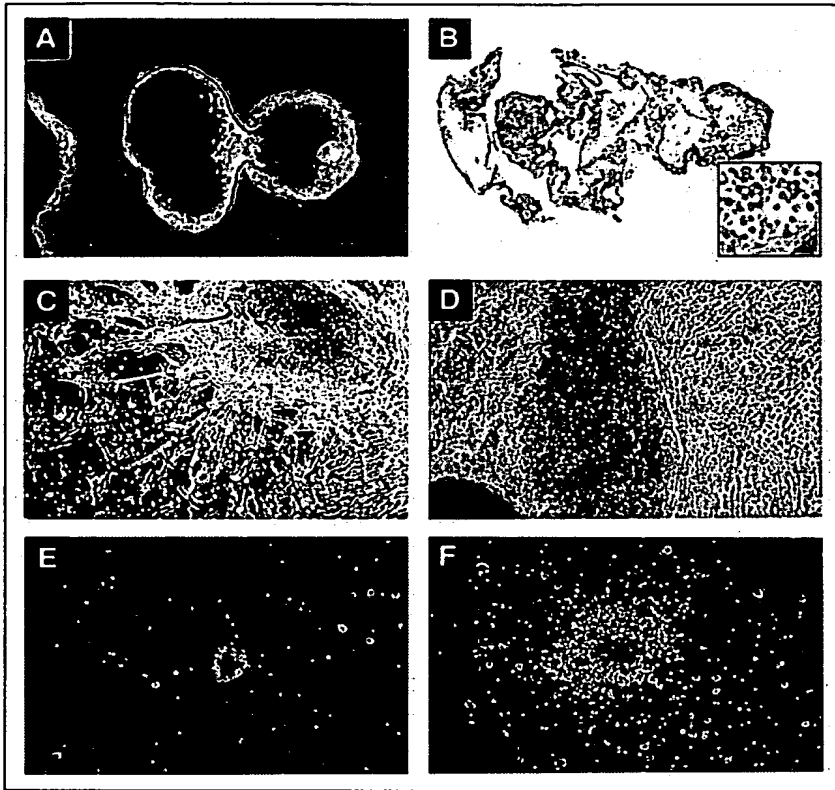
*Figure 2. In vitro differentiation of human ES cells under a variety of conditions. In suspension culture, human ES cells differentiate to EBs or multicellular aggregates resembling early embryos. A) A single H9 EB in suspension culture for 8 days demonstrating that complex, cystic EBs can be formed by this time (phase contrast, 100x); B) a single complex H1 EB in suspension culture for 14 days; most human EBs, regardless of the cell line, display the formation of extraembryonic tissue structures. Insert shows probable blood islands (hematoxylin and eosin staining, B, 100x; insert 300x). Following 8-14 days of suspension culture, H9 EBs transferred to gelatinized tissue culture plastic for further differentiation grow into confluent cell sheets containing a variety of differentiated cell types including C) neural cells (phase contrast, 40x), and D) pigmented and non-pigmented epithelial cells (phase contrast, 100x). After initial differentiation on S17 bone marrow stromal cells, and subsequent replating in methylcellulose-based media with hematopoietic growth factors, H1 cells can differentiate to E) BFU-E (darkfield phase contrast, 50x), F) colony-forming unit-granulocyte, macrophage (CFU-GM) (darkfield phase contrast, 100x), and CFU-M (not shown) colonies.*

[25-39]. From these experiments it is clear that ES cells induced to differentiate in culture follow many of the critical developmental stages found in the normal embryo, and are ultimately able to generate post-mitotic terminally differentiated cell types depending on the particular growth factor conditions.

As a result of their ability to differentiate into many different cell types, ES cells have been recognized as a valuable model system for studying the mechanisms underlying lineage specification during the early stages of mammalian development [25, 40, 41]. For example, by comparing downstream gene expression profiles between null mutant and wild-type ES cells, one can dissect the complex network of transcription factor genes regulating tissue-specific gene expression [42]. Also, in vitro culture provides a unique setting enabling control of the extrinsic cytokine or growth factor environment to study how these factors influence cellular differentiation [28, 31, 43]. Furthermore, in vitro differentiation of ES cells transduced with gene trap vectors can be used to discover novel developmentally regulated genes that are important in tissue-specific differentiation programs [44-47]. Thus, developmental pathways of cell lineages, which can be derived from ES cells, can be studied using this in vitro model system.

Recent studies demonstrate that human ES cells differentiating in culture are able to activate the expression of genes restricted to each of the three EG layers [18, 24, 43]. Human

EBs derived from the human ES cell line, H9, transcribed genes for α-fetoprotein, neurofilament 68kDa subunit, ζ-globin, and α-cardiac actin marking primitive endoderm, neuroectoderm, and mesoderm derivatives [24]. Differentiating cells acquired morphologies characteristic of neurons and cardiomyocytes [18, 24]. We have also performed human ES cell in vitro differentiation experiments. We observed that during subsequent development of plated EBs, cultures showed a variety of different morphologies, including rhythmically contracting cardiomyocytes, pigmented and non-pigmented epithelial cells, and neural cells displaying an exuberant outgrowth of axons and dendrites (Figs. 2C, D). Regions within the differentiated cultures also contained cells having a mesenchymal morphology.

*Schuldiner et al.* supplemented these data and showed that both undifferentiated human ES cells and differentiated EBs expressed receptors for a number of different soluble growth factors with established effects on developmental pathways in vivo [43]. Addition of each of these growth factors individually to the culture medium altered the expression profile of an array of tissue-restricted genes [43]. However, none of these growth factors directed differentiation exclusively to one particular cell type [43]. A better understanding of the epigenetic events regulating cell lineage commitment and differentiation should permit the focused use of soluble

growth factors to achieve lineage-restricted differentiation of human ES cells.

In other studies using human ES cells, we have used a co-culture method to promote hematopoietic differentiation. If human ES cells are allowed to differentiate on MEFs, hematopoietic colony-forming cells are not seen. In striking contrast, when the H1, H9, or clonal H1.1 human ES cell lines are co-cultured with irradiated mouse bone marrow stromal cells (S17 cells) in medium containing fetal bovine serum but no other exogenously added growth factors, they differentiate into a variety of cell types. The cell types derived under these conditions include cystic structures that resemble yolk sac, and cobblestone-type cells that are typical for hematopoietic precursors. CD34⁺ cells can be identified by flow cytometry, and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis shows induction of hematopoietic transcription factors. When human ES cells differentiated on S17 cells are harvested and placed in a methylcellulose colony-forming assay, a variety of hematopoietic colonies can be identified. These include colonies of erythroid, macrophage, granulocyte, and megakaryocyte cells (Figs. 2E, F). These data suggest that S17 bone marrow stromal cells either promote or support hematopoietic cell differentiation [48].

Endoderm lineages, such as pancreas and liver, are morphologically less distinct and more difficult to discern in ES cell cultures than blood or cardiac cells. This is one of the factors that has inhibited their detection among ES cell progeny. Differentiated ES cell progeny are able to express some endoderm and pancreas-restricted genes. Rhesus and mouse ES cells are capable of activating pancreatic and endoderm genes both in vitro and in vivo [38, 49]. Preliminary human ES cell gene expression studies show that differentiated derivatives of human ES cells can be induced to express endoderm genes, including hepatocyte nuclear factor 3 beta, and pancreatic islet genes including insulin, somatostatin, and glucagon (*JSO*, unpublished observations). Likewise, *Schuldiner et al.* recently presented RT-PCR gene expression data showing that differentiated progeny of human ES cells activate transcription of a variety of endoderm, liver, and pancreas-restricted genes [43]. However, it remains to be determined whether the initiation of pancreatic islet differentiation, as evidenced by gene transcription, will progress through a full differentiation program and lead to phenotypically adult islet cell populations with physiologic insulin secretion, as has been derived from mouse ES cells. Collectively, these data suggest that human ES cells can activate embryonic gene expression programs in culture and begin to differentiate into derivatives of all three EG layers.

## MULTILINEAGE DIFFERENTIATION IN VIVO

Although ES cells can differentiate to multiple embryonic and adult cell types in vitro, pattern formation or organogenesis

does not occur to a significant degree. Differentiation in the context of an in vivo environment, such as following injection into a host blastocyst or implantation into mice, unveils the full developmental potential of undifferentiated ES cell lines [3]. In this context, many of the normal features of tissue architecture are reproduced. For example, epithelia exhibit polarity, are enveloped by a basement membrane, and are surrounded by mesenchyme; complex tissue structures such as hair follicles, teeth, and gut are also formed. Human ES cells injected into severe combined immunodeficient mice form benign teratomas, with advanced differentiated tissue types representing all three EG layers (Fig. 3) [7]. Easily identifiable differentiated cells in human ES cell teratomas include smooth muscle, striated muscle, bone, cartilage, fetal glomeruli, gut, respiratory epithelium, keratinizing squamous epithelium, hair, neural epithelium, and ganglia (Fig. 3). Compared with human EC cell lines, human ES cell lines exhibit both more advanced and more consistent developmental potential. For example, the human EC cell line NTERA2 cl.D1 injected into immunocompromised mice forms teratocarcinomas containing simple tubular structures resembling primitive gut, neural rosettes, and tissue resembling neuropile [10].

Embryonic inductive events and complex epithelial-mesenchymal interactions control the formation of organized tissue structures during normal embryogenesis. These events and interactions begin to occur in teratomas but are less pronounced during in vitro differentiation. Unfortunately, the precise inductive events regulating embryonic pattern formation are still being elucidated and cannot yet be reliably reproduced in vitro. Because in vivo differentiation of human ES cells is more complete than in vitro differentiation, it would be useful to explore means to extract the cells or tissue of interest from the heterogeneous mix of tissues comprising teratomas or to direct differentiation in vivo to a particular lineage. Possible methods of achieving this include: A) adding specific combinations of chemical morphogens or growth factors [43, 50]; B) co-culturing or co-transplanting ES cells with inducer tissues or cells; C) implanting ES cells into specific organs or regions of animals; D) overexpressing tissue-specific homeobox transcription factor genes [51, 52]; E) selecting cells that activate a particular lineage-specific gene expression program [38, 53, 54], and/or F) isolating cells of interest based on fluorescence-activated cell sorting [55, 56]. Some of these methods have been explored to enrich in vitro ES cell cultures for a cell type of interest, but the application of these methods in combination with in vivo differentiation awaits future studies.

## DEVELOPING TRANSPLANTATION THERAPIES

Diseases that result from the destruction and/or dysfunction of a limited number of cell types, such as diabetes mellitus, in which pancreatic islet cells have been selectively
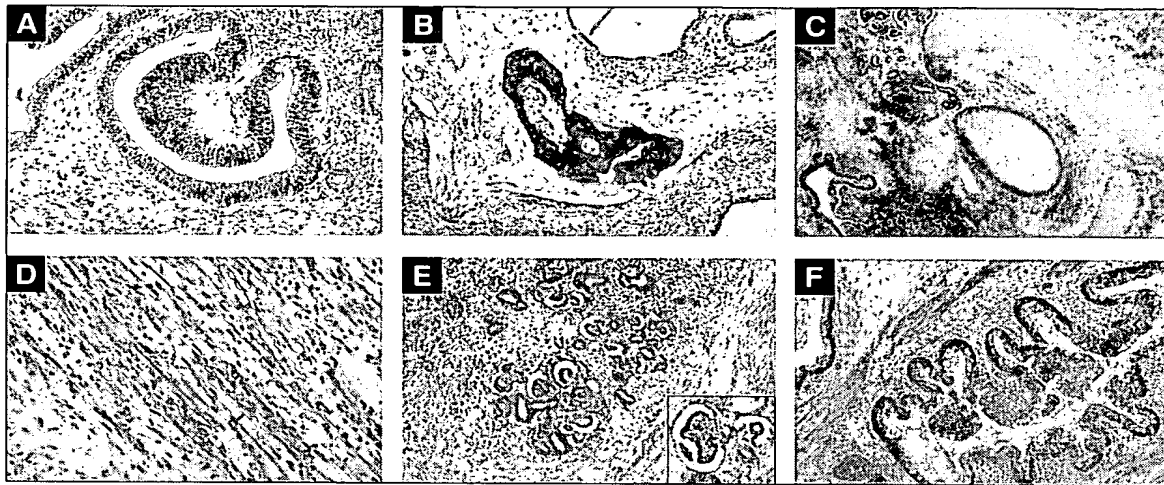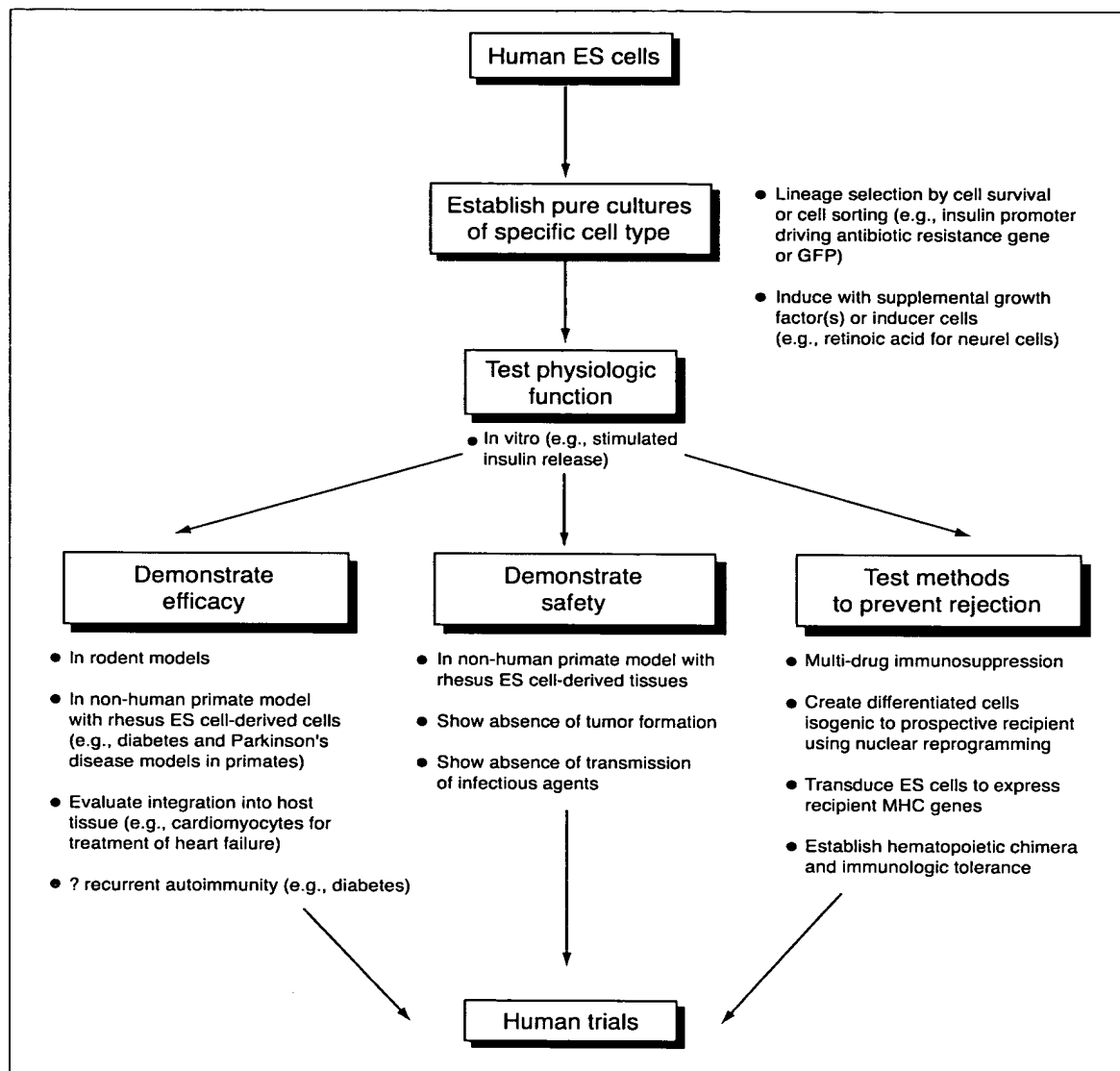
*Figure 3. Tissue derivatives of all three EG layers differentiated from human ES cells in vivo. Human ES cells injected into immunocompromised mice form benign teratomas. Present within these teratomas are advanced derivatives of ectoderm, such as A) neural epithelium (100×), of mesoderm, such as B) bone (100×), C) cartilage (40×), D) striated muscle (200×), and E) fetal glomeruli and renal tubules (100×; insert, 200×), and of endoderm, such as F) gut (40×). To some degree micro-architectural tissue relationships of complex organs can be reproduced in human ES cell teratomas. H1, H7C, H9, H13, and H14 cell lines, which produced the above teratomas, exhibit a similar range of differentiation. All photomicrographs are of hematoxylin- and eosin-stained sections.*

destroyed, or Parkinson's disease, which results from the destruction of dopaminergic neurons within a particular region of the brain, could be treated by the transplantation of differentiated derivatives of ES cells. Studies in animal models show that transplantation of either pluripotent stem cell derivatives, or fetal cells, can successfully treat a variety of chronic diseases, such as, diabetes, Parkinson's disease, traumatic spinal cord injury, Purkinje cell degeneration, liver failure, heart failure, Duchenne's muscular dystrophy, and osteogenesis imperfecta [38, 57-64].

Although considerable progress in human transplantation medicine has been achieved in recent years, several major obstacles still restrict more widespread application of cellular transplantation in the routine treatment of these conditions. The chief obstacles that face this field are the need for massive doses of immunosuppressive drugs to prevent rejection of the transplanted tissue and the scarcity of organs from human cadaver donors. In light of these obstacles, a human ES cell-based strategy could permit the generation of an unlimited supply of cells or tissue from an abundant, renewable, and readily accessible source. Moreover, by virtue of their permissiveness for stable genetic modification, ES cells could be engineered to escape or inhibit host immune responses.

The first step toward successful development of a stem cell-based therapy for human diseases is to establish that human ES cells are capable of differentiating to a particular cell type of interest and to purify this lineage from the mixed

population (Fig. 4). Unfortunately, the heterogeneous nature of development in culture has hampered the use of ES cell derivatives in transplantation studies. Rarely have specific growth factors or culture conditions led to establishment of cultures containing a single cell type [43, 65]. In fact, human pluripotent cell lines retain a broad pattern of multilineage gene expression despite the addition of specific growth factors [43, 65]. Furthermore, there is significant culture-to-culture variability in the developments of a particular phenotype under identical growth factor conditions. Given the broad range of lineages to which ES cells commit, derivation of a relatively homogeneous cell population will ultimately depend on selection from a mixed population of cells. One approach might involve using a tissue-specific promoter to drive a selectable marker such as an antibiotic resistance gene [38, 53, 54]. An alternative approach could involve transduction of a gene construct containing a tissue-specific promoter/enhancer controlling expression of a green fluorescence protein gene [66]. In this way, cells activating a lineage differentiation program of interest could be selected by fluorescence-activated cell sorting in much the same way that CD34+ hematopoietic stem cells are selected and sorted for stem cell transplantation. Both approaches would rely on the development of efficient gene transfer methodologies for human ES cells. A potential pitfall of these approaches is the concern for rejection of the transplanted cells which now express a foreign protein and the potential malignant transformation of genetically manipulated cells [67].

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                    │  Human ES cells  │
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                             │
                             ▼
              ┌─────────────────────────┐    ● Lineage selection by cell survival
              │  Establish pure cultures │      or cell sorting (e.g., insulin promoter
              │    of specific cell type │      driving antibiotic resistance gene
              └─────────────────────────┘      or GFP)
                             │
                             │                 ● Induce with supplemental growth
                             │                   factor(s) or inducer cells
                             ▼                   (e.g., retinoic acid for neurel cells)
                  ┌──────────────────┐
                  │ Test physiologic │
                  │    function      │
                  └──────────────────┘
```

● In vitro (e.g., stimulated insulin release)

**Demonstrate efficacy**

● In rodent models

● In non-human primate model with rhesus ES cell-derived cells (e.g., diabetes and Parkinson's disease models in primates)

● Evaluate integration into host tissue (e.g., cardiomyocytes for treatment of heart failure)

● ? recurrent autoimmunity (e.g., diabetes)

**Demonstrate safety**

● In non-human primate model with rhesus ES cell-derived tissues

● Show absence of tumor formation

● Show absence of transmission of infectious agents

**Test methods to prevent rejection**

● Multi-drug immunosuppression

● Create differentiated cells isogenic to prospective recipient using nuclear reprogramming

● Transduce ES cells to express recipient MHC genes

● Establish hematopoietic chimera and immunologic tolerance

**Human trials**

*Figure 4. Major goals in the development of transplantation therapies from human ES cell lines.*

Second, it will be critical to test and demonstrate that the differentiated cellular derivatives function in a normal physiologic way; i.e., that pancreatic islet cells exhibit normal glucose-responsive insulin secretion. Prior studies have demonstrated that many differentiated cell types derived from murine ES cells in vitro (e.g., cardiomyocytes and dopaminergic neurons) display a terminally differentiated, physiologically mature phenotype and do exhibit normal physiologic functioning in vitro and in vivo [36, 38, 68]. However, differentiated ES cell cultures can contain multipotent progenitors as well as terminally differentiated cells [69]. Because many fetal or embryonic tissues and multipotent progenitor cells are functionally immature, one cannot assume that all ES cell progeny will subserve normal cellular physiologic functions.

A third major milestone on the road to clinical trials will be to demonstrate efficacy in rodent and large animal models of disease. Rhesus ES cells and the rhesus monkey provide an excellent preclinical model for developing ES cell-based transplantation therapies and for testing strategies to prevent immune rejection. Indeed, for Parkinson's disease and diabetes mellitus, good models are already available in the rhesus monkey [70, 71]. Achieving a therapeutic result will mandate integration of the transplanted cells into the host tissue in a functionally useful form. For example,

replacing infarcted heart muscle or scar tissue with ES cell-derived cardiomyocytes will require that new muscle cells integrate with the existing muscle, contract in a coordinated and mechanically useful manner, and develop a new blood supply. Although complex structural integration would be essential for some cell transplants (e.g., neurons and cardiomyocytes), normal functioning of other ES cell-derived transplants will be more independent of such complex tissue interactions (e.g., islet cells and hematopoietic cells).

Fourth, the possibility arises that transplantation of differentiated human ES cell derivatives into human recipients may result in the formation of ES cell-derived tumors. From in vivo differentiation studies, it is clear that if undifferentiated rhesus or human ES cells are not rejected after implantation into host recipient animals, then a benign teratoma can result [6, 7]. These tumors are not metastatic, and do not rapidly kill the host animals. Tumor growth in immunodeficient animals appears to be dependent on the presence of a stem cell population in undifferentiated cultures. Thus, as ES cells are allowed to fully differentiate into post-mitotic, terminally differentiated derivatives, they should deplete the undifferentiated stem cell pools, thereby reducing the probability of uncontrolled tumor growth. In fact, from a limited number of short-term studies, it appears that transplanting differentiated progeny derived from murine ES cells into adult rodents does not result in significant tumor formation [38, 53, 57, 72]. However, these studies were not specifically

designed to address this question, and as such, they lack sufficient animal numbers and long-term surveillance to allow firm conclusions. If tumor formation does depend on persistence of a stem cell population, then one could design a transgenic methodology to eliminate residual minority stem cells from differentiated ES cell cultures, possibly based on negative selection of Oct 4-expressing cells. Use of a positive selection transgene to achieve lineage-directed differentiation would also reduce the risk of tumor formation by selecting against the remaining undifferentiated, proliferating stem cell population. Irrespective of the persistence of stem cells, the possibility for malignant transformation of the derivatives will also need to be addressed. Ultimately, as the potential for tumor growth is a major safety consideration, a fail-safe method to prevent tumor growth may need to be developed. Once devised, strategies could be tested in a preclinical rhesus monkey model, using rhesus ES cell-derived cells, prior to embarking on human clinical trials.

## PREVENTING IMMUNOLOGIC REJECTION OF TRANSPLANTED CELLS

A fifth consideration is the prevention of immune-mediated rejection of the human ES cell-derived cellular graft (Figs. 4 and 5) [73]. Currently available multidrug immunosuppressive regimens are effective in preventing rejection in most recipients of solid organ transplants. Even with a modest level of efficacy, a therapy brought to clinical trials for a disease
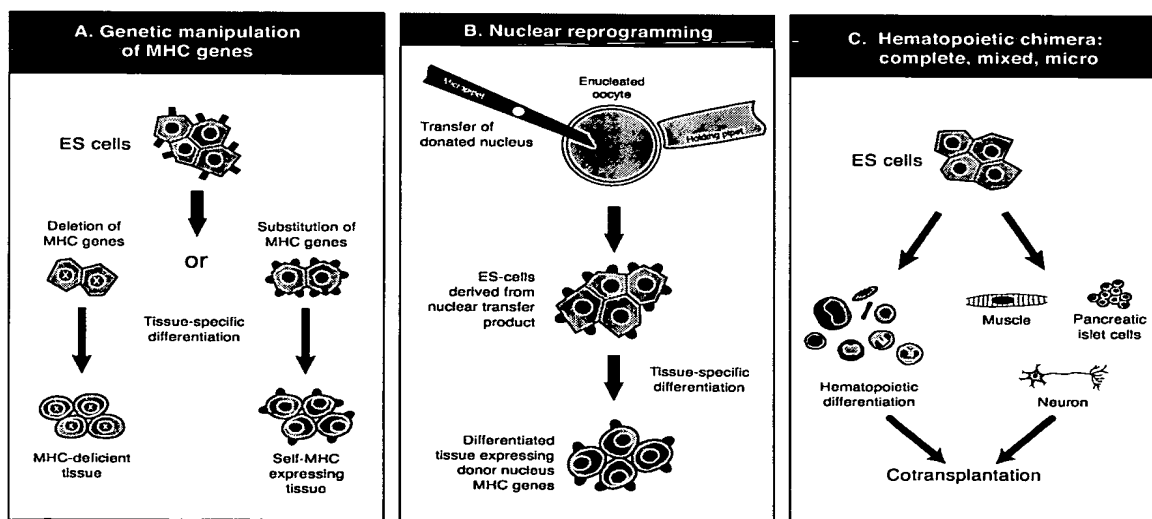


Figure 5. Possible ways to circumvent immune-mediated rejection of tissue transplants derived from human ES cells. A) ES cells can be genetically altered to either eliminate foreign MHC genes or replace foreign MHC genes with ones specifically matched to a particular transplant recipient. B) In nuclear reprogramming, a nucleus from a somatic cell of an individual can be reprogrammed by transfer to an enucleated oocyte. An embryo established by nuclear transfer could be used to derive an ES cell line that expresses all histocompatibility antigens and other nuclear genes identical to those of the person from whom the somatic cell was obtained. C) Hematopoietic stem cells and a second tissue generated from the same parental ES cell line could be transplanted simultaneously or successively to the same recipient after administration of a nonmyeloablative conditioning drug regimen. This would create a hematopoietic chimera, thereby establishing immunologic tolerance to the second tissue.

with no other available treatment might still be considered a viable option, particularly if there were few repercussions of a failed transplant for the patient other than being returned to their baseline disease state. Unfortunately, however, currently used immunosuppressive drugs are far from ideal and are associated with numerous complications including wound healing, opportunistic infections, drug-related toxicities, skin malignancies, and low-grade lymphomas called post-transplant lymphoproliferative disorders. Instead, human ES cells could be genetically manipulated to reduce or eliminate immune-mediated rejection so that lifelong pharmacologic immunosuppression would not be required.

One potential method for limiting the immune response is to decrease the immunogenicity of transplanted cells. Homologous recombination has been used to "knock-out" major histocompatibility complex (MHC) class I and class II molecules in mouse ES cells [74]. However, MHC class I- and class II-deficient skin grafts are still rejected, possibly on the basis of indirect allo-recognition-mediated rejection and/or natural killer cell-mediated destruction [74]. Thus, in addition to deleting foreign MHC genes, it might be necessary to "knock-in" the desired MHC genes, so that ES cell-derivative transplants are seen as "self" by the prospective recipient [75, 76]. Alternatively, genes for immunosuppressive molecules such as Fas-ligand could be inserted into ES cells, or important immune-stimulating proteins, such as B7 antigens or CD40-ligand, could be deleted from ES cells [77, 78]. Irrespective of the method used, the ability to stably integrate genetic modifications into ES cells provides an advantage over using adult somatic cells, which are less reliably genetically altered.

Precisely how immunogenic is a cellular or tissue transplant from human ES cells? This may be a relevant question to ask as scientists attempt to engineer ES cells to reduce their immunogenicity. The immunogenicity of tissues is generally correlated with the MHC antigen expression profile and the relative abundance of antigen-presenting cells (e.g., dendritic cells) within the tissue. The MHC expression profile of human ES cell derivatives will depend on the degree of differentiation and/or the specific cell type derived. For example, adult somatic cells normally only express MHC class I antigens, whereas B cells, macrophages, and dendritic cells typically express both class I and class II antigens. Furthermore, whereas most adult organs and tissues harbor immunostimulatory dendritic cells and vascular endothelial cells, these normal tissue components would be expected to be absent from ES cell-derived cellular or tissue transplants. The specific removal of antigen-presenting cells from solid organ transplants generally increases graft survival [79, 80]. Consequently, some ES cell-derived tissues may be rather inert immunologically, while others, like hematopoietic stem cells, may be as

immunogenic as normal adult tissues. Therefore, human ES cell-derived transplants may in some cases provide an inherent immunologic advantage compared to human cadaver tissue transplants.

Nuclear transfer technology may provide a more precise means to prevent rejection of transplanted cells (Fig. 5). This technique would lead to ES cell-derived cells that are an exact genetic match to the recipient. In this way, there should be minimal host immune response since all nuclear genes, including major and minor histocompatibility loci, would be seen as "self." Here, a nucleus would be extracted from a normal somatic cell of a patient, say from a skin biopsy, and then injected into an enucleated oocyte. Oocyte cytoplasm has the ability to reprogram differentiated nuclei, and as such, would reestablish an embryonic gene expression program in the chromatin of the somatic cell nucleus. A blastocyst developing from this oocyte would be a source for the derivation of a new ES cell line, which would be genetically matched for each nuclear gene of the patient. In this setting, the potential immune-mediated destruction of the graft would be limited to minor antigen differences derived from mitochondrial genes or to autoimmune processes, such as diabetes. Extending nuclear transfer technology to achieve the fusion of an entire cell with an enucleated oocyte might eliminate some of the remaining genetic differences that would exist between ES cell derivatives of nuclear transferred embryos and the prospective patient. In fact, in cows and mice, investigators have successfully combined nuclear transfer technology and ES cell derivation to establish transgenic ES cell lines from reprogrammed somatic cell nuclei [81]. Clearly though, the generation of human embryos by nuclear reprogramming to create novel human ES cell lines would be exceptionally controversial. Furthermore, the poor availability of human oocytes, the low efficiency of the nuclear transfer procedure, and the long population-doubling time of human ES cells make it difficult to envision this becoming a routine clinical procedure even if ethical considerations were not a significant point of contention. By studying how oocyte cytoplasm mediates nuclear reprogramming in these animal models, it might be likely that nuclear reprogramming could be achieved by other methods, thereby obviating the need for human oocytes.

Establishing hematopoietic chimerism is another potential means of preventing rejection of transplanted cells (Fig. 5). There are now many patients who have undergone bone marrow transplantation and subsequently received a solid organ transplant (typically a kidney) from the same donor as the bone marrow [82, 83]. In these circumstances, no immunosuppression is required for the solid organ transplant because the recipient's lymphocytes are from the same donor and have rendered the recipient immunologically tolerant. However, success of a bone marrow or hematopoietic

stem cell transplant has generally required highly toxic immunosuppression. More recently, strategies have been designed which are significantly less myelotoxic while still permitting engraftment of donor hematopoietic stem cells [84]. This relatively mild treatment can permit long-term engraftment and could potentially allow successful solid organ transplantation in humans without prolonged immunosuppressive therapy. By using the same ES cell lines to derive both hematopoietic stem cells and other lineages, it may be possible to initially achieve hematopoietic chimerism followed by engraftment of a second cell type. Based on the principles described above, a second lineage should not be rejected as it would be regarded as "self" by the chimeric patient's bone marrow and immune system, which were derived, in part, from the same ES cell line. No long-term treatment with potentially toxic drugs would then be required. Ultimately, the rhesus monkey model will be essential for testing these and other strategies to prevent immune rejection.

Human ES cells fulfill all of the criteria of pluripotent ES cells. They are capable of indefinite self-renewal and multi-lineage differentiation, both in vivo and in vitro. This dual property provides the rationale for developing human ES cells as a basis for therapeutic tissue replacement. Not only do transplantation therapies based on ES cell-derived tissues offer the potential of overcoming limited tissue supplies, but they also present the exciting possibility of being able to perform tissue transplants with minimal or no immunosuppression.

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# Embryonic Stem Cell Models of Development

K. SUE O'SHEA*

Pluripotent mouse embryonic stem (ES) cell lines have provided a means to analyze gene function in development via gene targeting. At the same time, they provide an opportunity to directly probe gene function by assessing the in vitro differentiation capacity of the ES cells themselves. In addition to providing direct data on lineage decisions not accessible in the complex three-dimensional milieu of the early mouse embryo, controlled differentiation of ES into specific lineages may provide a source of cells for transplantation and gene therapy. *Anat. Rec. (New Anat.): 257:32–41, 1999.* © 1999 Wiley-Liss, Inc.

With the mouse genome project well underway, the goal of understanding gene function in mammalian development appears a real possibility. Technological advances for introducing deletions, human chromosomes, designer genes, inducible expression constructs, and marker genes into the mouse germline have revolutionized our ability to probe the role of a particular gene in development. These studies have many (some surprising) leitmotifs: families of signaling molecules play multiple, sequential roles in development; inhibitors may play as an important role in development as inducers; they have indicated a high degree of gene conservation. Gene targeting was made possible by the derivation in 1981 of the embryonic stem cell, which can be removed from the inner cell mass of the blastocyst, maintained (and manipulated) in vitro, then resumes development when reintroduced into a blastocyst. In addition to

their use in gene targeting, these pluripotent cell lines provide a tremendous resource for studies of gene expression and in vitro differentiation.

## MULTIPOTENT CELL LINES: ES, EC, AND EG

Embryonic stem (ES) cells are derived from the preimplantation blastocyst inner cell mass[16,30] (Fig. 1). When passaged in vitro under conditions that inhibit differentiation—e.g., on the sur-

> In addition to their use in gene targeting, these pluripotent cell lines provide a tremendous resource for studies of gene expression and in vitro differentiation.

face of embryonic fibroblasts or in complete medium supplemented with leukemia inhibitory factor (LIF)—ES retain their ability to develop into all cell types, including germ cells. They exhibit apparently unlimited replication potential, which may be related to the extremely low level of genomic DNA methylation (which imparts an additional level of on-off regulatory

control) in the blastocyst. ES cells have been most extensively employed to create null mutations in the mouse via gene targeting and homologous recombination. Although gene "knock out" has revolutionized our ability to probe and understand the mammalian genome, differentiation of these multipotent cell lines is also a powerful model of the early events involved in development and lineage specification.

In terms of their gene expression pattern, ES resemble the late inner cell mass (ICM) more than cells of the epiblast, although each line may have individual characteristics. ES cells have been derived from a number of mouse strains—including mutant strains (e.g., *t* mutants). They vary considerably in their ability to colonize the germline, possibly due to individual cell cycle, cell surface, or other genetic differences, or pre-patterning of the mouse epiblast (e.g., Gardner and Brook[19]), all of which may limit their differentiation capabilities. The most successful lines have been derived from 129 strain mice. ES-like (ESL) cells have been derived from other species, including rats, chickens, primates, and very recently human embryos,[50] but when reintroduced into blastocysts, none has successfully colonized the germ cell line—the gold standard of totipotency.

ES share many characteristics (and important differences) with tumor de-

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rived embryonal carcinoma (EC) cell lines. EC are typically derived from transplanted postimplantation embryos, grafts of epiblast or genital ridges, or from tumors arising spontaneously from germ cells. Studies of the pattern of gene and protein expression have indicated that unlike ES, EC resemble the epiblast more than ICM. Not surprisingly then, most EC lines exhibit restricted developmental options, such as egg cylinder ectoderm (F9), or fibroblast, muscle and neural lineages (P19). Both ES and EC cells proliferate extensively and express stem-cell marker genes (e.g., Oct 4, Rex 1, SSEA1, Forssman antigen). They have multipotential development capacity, and can form teratocarcinomas (undifferentiated tumors) and chimeras (embryos derived from EC and normal ICM). However, EC have a low capacity to contribute to the germline,[49] and often continue to proliferate when introduced into the embryo, indicative of their transformed, tumorigenic nature.

A third class of pluripotent cell line has been developed by "reprogramming" primordial germ cells (PGC) into pluripotent embryonic germ (EG) cell lines. Marking studies have indicated that mouse primordial germ cell precursors are present in the proximal epiblast near the extraembryonic ectoderm. PGCs then migrate into the extraembryonic mesoderm outside the amniotic fold of the primitive streak-staged embryo, then to the genital ridge where they enter mitotic and meiotic arrest in the primitive testis and ovary. Culture of PGC in a cocktail of cytokines (LIF, bFGF and particularly Steel factor[27]) results in "de-differentiation" of the postimplantation PGCs into cell lines with some characteristics similar to ES, thus the name EG (embryonic germ) cells. EG cell lines have not been obtained from genital ridges after midgestation and are most efficiently derived from posterior regions of day 8–8.5 embryos, suggesting that the growth factors may block the differentiation of PGCs to immature germ cells.

EG cells resemble immature germ cells in that like germ cells, they lack genomic imprinting marks (specific patterns of DNA methylation on certain genes that are inherited from either the mother or the father and
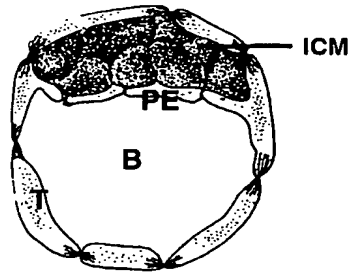


**Figure 1.** Organization of the early blastocyst. The inner cell mass (ICM) is located at one side of the fluid filled blastocoele (B). Primitive endoderm (PE) cells are forming at the base of the ICM, and the entire embryo is surrounded by trophoblast (T).

affect the proper differential expression of some maternal and paternal genes); in ES, the parental imprint is largely maintained. They have cell-cycle distribution characteristics similar to ES and EC, as well as the ability to differentiate in vitro into several cell types, including cardiac and skeletal muscle, as well as neurons.[43] EG can contribute to chimeras,[27] and to the germline[48] when they are introduced into host blastocysts. The recent isolation of pluripotent human EG-like cell lines derived from human fetal gonad[46] suggests that this may be an important source of pluripotent human cell lines for similar studies.

## DIFFERENTIATION PARADIGMS

Traditionally, studies of in vitro differentiation have been carried out using EC cell lines. However, since most exhibit restricted developmental potential, ES have largely replaced EC in these investigations. Because they are pluripotent, and can be extensively modified to alter their pattern of gene expression, ES cell lines have potential in studies aimed at elucidating the role of a particular gene, cytokine or growth factor in differentiation. They have also been used to isolate unique genes involved in cell-type specific differentiation and to study the cascade of gene expression during the development of a particular lineage or tissue. Controlled differentiation of these cells would also provide a theoretically unlimited source of material for biochemical and genetic analyses. In addition, because ES are derived prior to implantation, certain immune-related

cell-surface proteins (e.g., class I products of the major histocompatibility complex) are not yet expressed, which makes ES cells a potentially rich source of material for transplantation and gene therapy approaches.

The model systems that have been employed for ES differentiation are outlined in Figure 2.
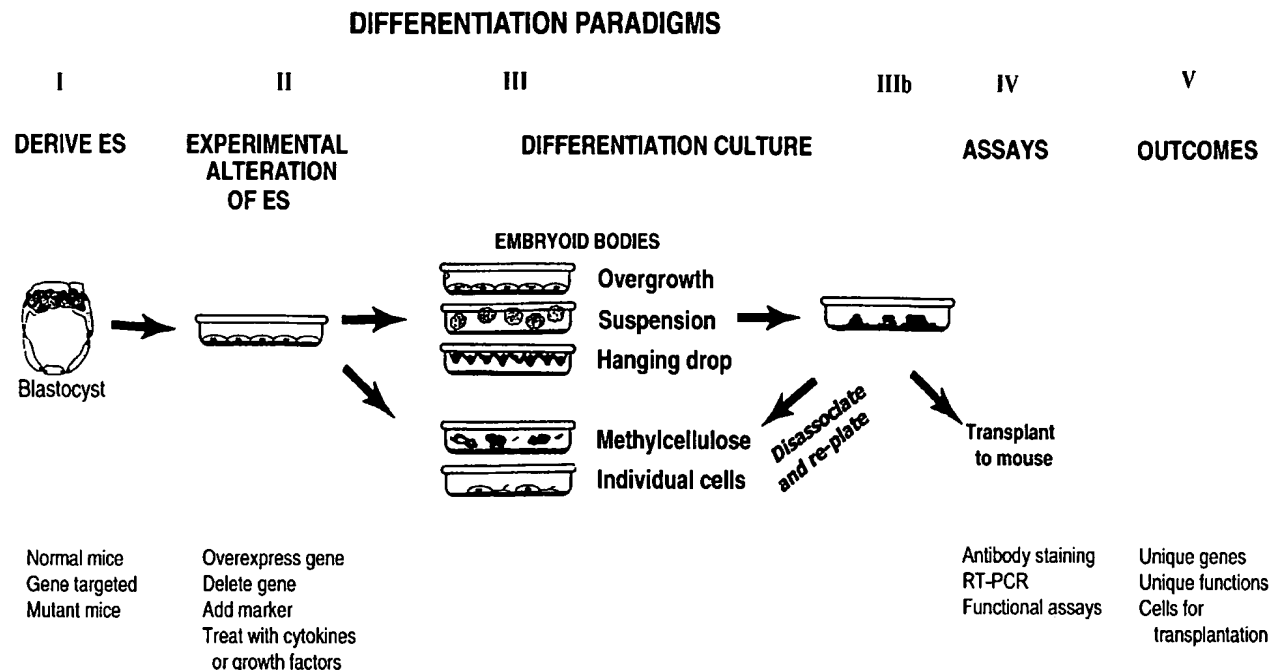
## Formation of Embryoid Bodies

Differentiation culture of ES is typically carried out by removing LIF from the medium and allowing cultures to overgrow, by plating cells in suspension on non-adhesive substrates (bacteriological-grade culture dishes), in "hanging drop" (to control cell number and force cell-cell contact), or in

> **Because ES are derived prior to implantation, certain immune-related cell-surface proteins are not yet expressed, which makes ES cells a potentially rich source of material for transplantation and gene therapy approaches.**

low-substrate adhesion conditions such as in methylcellulose (Fig. 2III). After 4–8 days in vitro, cells aggregate into irregular clumps, which, because they grossly resemble early embryos—endoderm exterior, mesoderm and ectoderm interior, surrounding a large cystic yolk sac–like cavity—have been termed "embryoid bodies" (EB; Fig. 3B). The resulting EB are then either plated directly on adhesive tissue culture plastic, disaggregated and then plated, or transplanted to brain, subcutaneously, to kidney capsule or chick chorioallantoic membrane. EBs have been widely employed to study hematopoietic differentiation, cardiomyocyte development, and neuronal differentiation, as well as many cell-biologic events including epithelial cavitation

# DIFFERENTIATION PARADIGMS

| I | II | III | IIIb | IV | V |
|---|----|-----|------|----|----|
| **DERIVE ES** | **EXPERIMENTAL ALTERATION OF ES** | **DIFFERENTIATION CULTURE** | | **ASSAYS** | **OUTCOMES** |

**EMBRYOID BODIES**

Overgrowth

Suspension

Hanging drop

Methylcellulose

Individual cells

Transplant to mouse

Blastocyst

| | | | | |
|---|---|---|---|---|
| Normal mice | Overexpress gene | | Antibody staining | Unique genes |
| Gene targeted | Delete gene | | RT-PCR | Unique functions |
| Mutant mice | Add marker | | Functional assays | Cells for |
| | Treat with cytokines or growth factors | | | transplantation |

**Figure 2.** Various paradigms have been employed to study the differentiation potential of embryonic stem (as well as EC and to a lesser extent EG) cell lines. I: ES may be derived from normal mice, mice carrying a marker gene (e.g., green fluorescent protein, ROSA mice), from mice carrying a spontaneous (e.g., Splotch mutant) or a mutation engineered by gene targeting and homologous recombination. II: Blastocysts are grown in vitro and clumps of rapidly proliferating cells (ICM) removed, dissociated and an ES line established. At this stage it is possible to carry out gene targeting experiments (gain- or loss-of-function alterations) and/or ES can be treated with a growth factor or cytokine of interest. III: Two basic paradigms for ES differentiation have been employed. In the first, indirect method, cells are removed from inhibitors of differentiation (LIF) and simply allowed to overgrow—resulting in aggregates of rapidly dividing ES which lift off the substrate to float and continue differentiation. In suspension and hanging drop culture, cells are removed from their substrate, disaggregated and either grown in non-adhesive tissue culture dishes, or 100–1,000 ES cells in microliter drops of culture medium are placed in the lid of a culture dish. After several days of aggregation culture, embryoid bodies are exposed to a chemical inducing agent (typically retinoid acid or DMSO). After an additional 2–4 days in vitro, EB are plated on adhesive culture dishes (IIIb) for differentiation. In a derivative method EB are dissociated, then cells plated in defined medium. The single step protocol relies on minimal cell-cell contact in methylcellulose (employed nearly exclusively for hematopoietic differentiation) or on exposure to a differentiating agent or gene and plating as individual cells on adhesive substrates. IV: Unraveling the precise role of a gene or treatment requires careful analysis. Cell type specific antibody staining with morphological identification has been extensively employed to identify cell types ± RT-PCR, northern analysis, and western blotting. Functional assays such as channel function in neurons, gap junction coupling have also been employed. V: Outcomes are many and range from obtaining new genes by subtractive hybridization of RNAs from I v II, or I v III, gene trap following growth factor exposure, determining the functional role of a particular gene in cell type specific differentiation (e.g., MyoD in muscle cell development). Cell differentiation into a single lineage, or separation by e.g., FACS will also provide partially purified cells for transplantation, gene delivery.

and gap junction communication. A step-by-step description of EB differentiation (with a focus on hematopoietic differentiation) can be found in Wiles.[53]

EB differentiation is a relatively simple method to determine the effects of genetic alteration on differentiation. For example, does overexpression of the MyoD gene in ES cells result in an increased number of muscle cells in the EB? When gene deletion is lethal early in embryonic development, EB may be the only approach to assess differentiation (e.g., beta-1 integrin knockout; Fassler and

Meyer[17]). However, both gene copies, or alleles, typically must be targeted in these studies, to create "homozygous null" ES. Gene targeting in ES is a much more precise method to deplete a gene or gene product from a cell than using antisense oligonucleotides, and has the added advantage that the targeted gene (or gene family member) can be reintroduced to rescue a phenotype. Finally, EB also provide a source of cells not otherwise available for study. For example, cardiomyocytes derived from early embryos fail to survive in vitro, unlike those derived by EB differentiation.

Although the pattern of gene expression during EB differentiation is similar to that in the early embryo, EB contain increased numbers of endodermal and mesodermal derivatives at the expense of neural and muscle cells, indicating that factors required for the normal differentiation of these lineages are absent (or reduced) in embryoid bodies.[39] With early differentiation, each cell type in an EB secretes its own panel of growth and differentiation factors and the resulting differentiation of the aggregate is "chaotic," often resembling a teratoma, with multiple lineages present in a jumble (Fig.
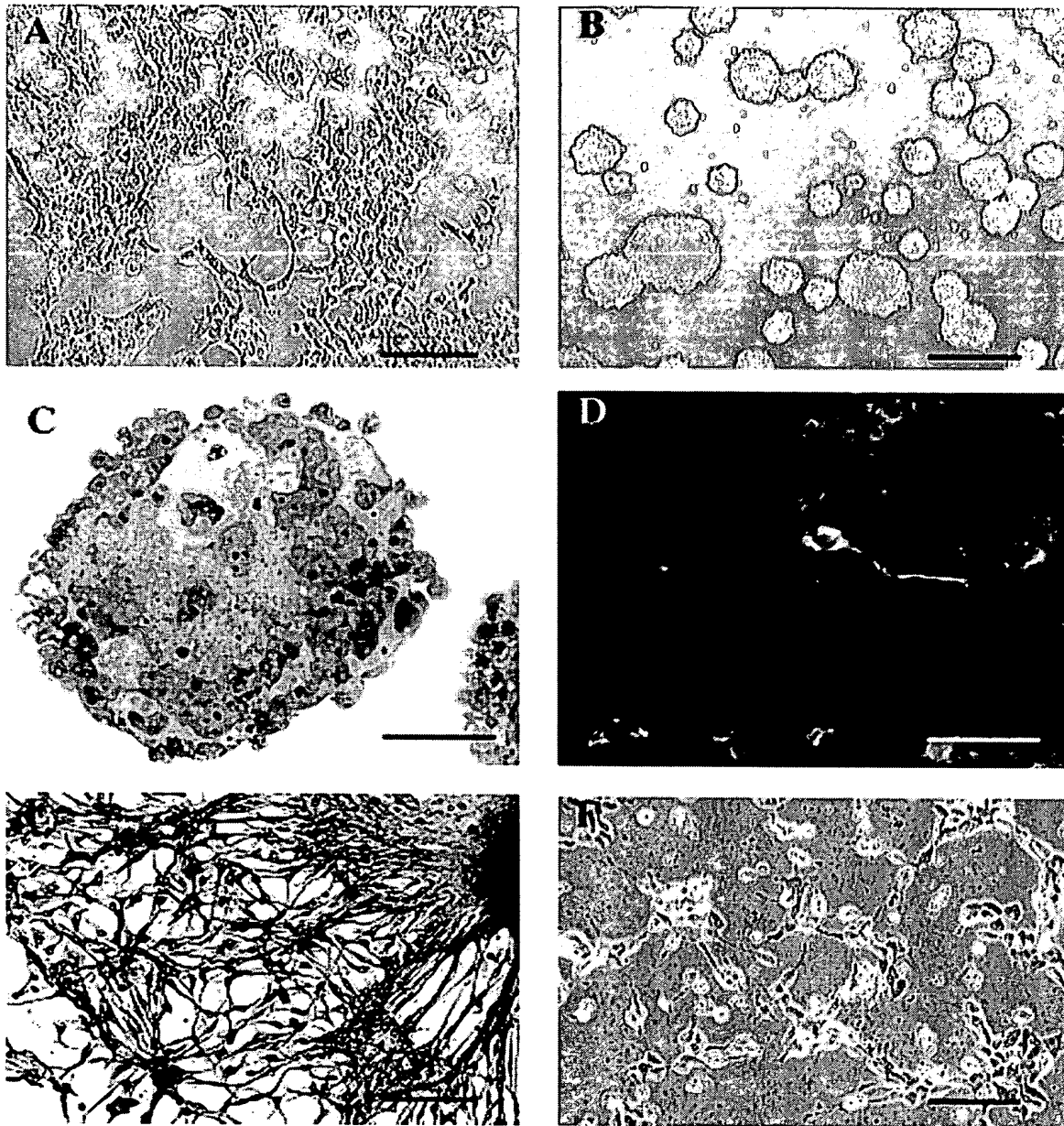
**Figure 3.** A: Phase contrast micrograph of D3 ES growing in a gelatin coated flask in complete medium (20% serum) containing LIF. B: EB growing in suspension culture in complete medium without LIF. A layer of flattened endoderm surrounds the aggregated ES. After only 4 days in vitro, the larger EB are forming fluid-filled central cavities. C: EB were fixed, embedded in resin and sectioned at 2 μm to illustrate the unorganized jumble of differentiating cells within an early EB. D: Other EB were fixed, frozen sections cut (8 μm), and exposed to cell type specific antibodies (in this case anti-neurofilament medium, a neuronal marker, followed by secondary antibody—FITC) to indicate differentiation within the EB. E: Neuronal differentiation is extensive when EB are treated with RA and plated on laminin coated dishes in defined medium. Anti-neurofilament antibody staining, secondary antibody—DAB. F: Direct differentiation of ES, in this case following the expression of a noggin cDNA and differentiation in defined medium on a laminin coated substrate. Neuronal differentiation is seen after 24 h, and is extensive by the 48 h timepoint illustrated here. Scale bars B = 250 μm; others = 50 μm.

3C) rather than the precise, controlled organization of the early embryo.

Differentiation of EB is typically carried out in high serum conditions, which can obscure the effects of growth factors being tested. In addition, induction of differentiation is often accomplished by agents such as DMSO, db-cAMP, or retinoic acid (RA), whose physiological relevance may be negligible. Interesting developmental questions are raised, however, by EB biology: What is the role of cell-cell contact in differentiation, as well as the role of inside-outside positioning (like the transition of ICM to epiblast) in cell type specification? Is it the absence of spatial positioning information provided by uterine vessels that causes cells in EB to fail to organize as tissues?

## Non-Aggregation (Direct) Differentiation

To date, most models of ES differentiation require an aggregation step; however, a few studies have reported direct differentiation of ES into a particular cell type, including lineages from all three germ layers (e.g., Rathjen et al.[39]).

A relatively new approach to studying the direct differentiation of ES is to transfect them so they constitutively express lineage determination genes. For example, ES transfected to express the muscle cell determination gene MyoD1 form a high percentage of skeletal muscle cells; expression in ES of neural determination genes NeuroD2 or NeuroD3 produces robust neuronal differentiation. Another approach to enrich for a particular cell type is to express an antibiotic resistance gene via a promoter whose expression is restricted to the desired lineage. For example, to obtain ES cells from the non-permissive CBA strain, transgenic mice were constructed in which the coding region of the stem cell transcription factor OCT3/4 was linked to the neomycin resistance gene. ICM from transgenic embryos were grown in medium containing high levels of antibiotic so that as they differentiate, cells not expressing OCT3/4 are killed by antibiotic selection, producing an undifferentiated ES line.[31] As more is known about genes involved in lineage determina-

tion, the directed differentiation of ES will provide a more homogeneous population of derivatives for analysis and for transplantation.

## ES DIFFERENTIATION

### Cell Biology

ES cells have been employed extensively in studies in which deletion of structural genes has proved to be embryonic lethal. For example, in the absence of beta-1 integrin (ITGB1), embryos die around implantation,[17] precluding careful analysis of the role of ITGB1 in cell migration, basement membrane formation, etc. ITGB1-null ES cells exhibit abnormal adhesion, migration and cellular morphology in vitro, and when differentiated as EB the inability of ITGB1-null ES to assemble a basement membrane results in a lack of keratinocyte differentia-

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tion, delays myoblast differentiation, and when transplanted into nude mice, tumors either fail to form or are very small and disorganized.[7]

Deletion in ES cells of genes involved in the formation of focal adhesions, such as vinculin,[10] has striking effects on their shape, spreading and adhesion. Somewhat surprisingly, deletion of focal adhesion kinase has little effect on differentiated derivatives of ES,[21] and EB formed from ES deficient in the tight junction protein occludin have functional tight junctions.[45] Other surprising results have been obtained in EB differentiation studies, e.g., deletion of both alleles of the mouse keratin 8 gene results in failure of dimeric cytokeratin filaments to form, but epithelial organization is normal.[5] Although ES are not a particularly invasive cell type, when one allele of the tissue inhibitor of

metalloproteinases (TIMP1) gene is inactivated, and cells differentiated to a mesenchymal phenotype using RA, they efficiently invade a matrigel substrate.[2] Use of null ES has also allowed rescue experiments by the re-expression of the deleted gene or a related gene. For example, E-cadherin null ES fail to aggregate as embryoid bodies. When R-cadherin, but not other cadherins, is expressed in these cells, formation of skeletal muscle (and to a lesser extent, epithelium) was specifically (and surprisingly) rescued.[44]

Because of their apparently unlimited proliferation potential, ES are a natural model system to examine genes associated with normal growth and proliferation, cell cycle control genes, oncogenes, receptor tyrosine kinases, phosphatases, and genes involved in the LIF signaling pathway. Interestingly, many proto-oncogenes (e.g., c-myc, c-jun, c-fos) are not required for differentiation in EB, but tumorigenesis is severely affected when EB from oncogene-deficient ES are transplanted into nude mice.

The patterns of expression of a number of regional developmental control genes (e.g., Hox, Pax, Pou, and Oct) have been examined in differentiating EB. Expression of these genes generally follows the differentiation of EB, and has allowed research on their regulation by chemical inducers. These studies have generally not led to increased understanding of the role of these genes in differentiation, possibly because of the lack of anterior-posterior and dorsal-ventral positional information in EB.

ES have been exposed to a range of differentiating agents, including: RA, DMSO, HMBA, growth factors and cytokines (LIF, TNFα, activin A, BMP-2,4, insulin, NGF, bFGF, CNTF, T3) and panels of hematopoietic growth factors with the goal of inducing/ modifying differentiation or to examine alterations in gene expression induced by these agents, with mixed results. These specific differentiation paradigms will be briefly described in the following sections on tissue type-specific differentiation.

### Genetic Investigations

Genetic research has also been carried out using ES differentiation para-

digms. The study of imprinting in parthenogenetic—or gynecogenetic (two maternal chromosome sets) and androgenetic (two paternal chromosome sets)—ES lines during their differentiation has provided a mechanism to examine the role of DNA methylation and somatic maintenance of imprinted genes.[3] Parthenogenetic ES fail to form trophectoderm or primitive endoderm, but contribute extensively to tissues in the early embryo, where they are gradually removed by apoptosis during mid-gestation—particularly in skeletal muscle. (Parthenogenesis has been studied extensively in the mouse, where an unfertilized oocyte can be induced to develop in the absence of a genetic contribution from sperm.) Other conditions of abnormal chromosome number or constitution (aneuploidy) are typically midgestation lethal, but differentiation of ES lines derived from aneuploid embryos provides the opportunity to examine differentiation, apoptosis and the selective differentiation ability of the aneuploid cells.

Mutations—both environmental (X-ray and chemical mutagens), as well as those caused by DNA insertions—have been introduced into ES to identify genes required at various stages of embryogenesis (e.g., Robertson et al.[41]). For example, the retroviral insertion 413.d mutant led to the identification of the critical mouse gene, nodal. When lethality occurs early in development it is not possible to determine if the gene might also participate in later tissue differentiation, so ES have been derived from the ICM of embryos homozygous for the mutation and differentiated in vitro. Study of chromosomal deletions is also possible using ES obtained from embryos with e.g., a deletion in the albino gene locus. Such embryos fail to form mesoderm, leading to the identification of the mesoderm deficient gene locus, msd.

A more efficient approach used to identify new genes of unknown function is to employ a technique known as gene trapping. ES are transfected with a promoter-less expression construct containing an antibiotic resistance gene and a marker gene (i.e., the beta-galactosidase gene) which will be expressed only if the construct inserts into an active transcriptional unit. By

antibiotic selection, genes of interest can be identified and sequenced. Their pattern of expression can then be determined via expression of the reporter gene, and the effects of a mutation can be rapidly monitored in EB before generating embryos (e.g., Evans et al.[15]).

## LINEAGE SPECIFICATION

Signaling molecules, cell-cell contact and positional information result in site specific differentiation in the early embryo, with proximal epiblast forming surface and neural ectoderm and distal epiblast forming mesoderm. Given their similarity to ICM/epiblast, ES cells are being used to identify factors that may motivate these very early lineage choices. Much of this work has examined factors identified in *Xenopus* patterning, particularly BMPs and activin, which produce ventral/posterior or anterior/dorsal meso-

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Mutations have been introduced into ES to identify genes required at various stages of embryogenesis.

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derm from ES cells, respectively.[22] In defined medium, in the absence of signaling molecules, ES differentiate into neuroepithelium—like cells, as determined by expression of Pax 6.[54] ES themselves express a number of growth factors and cytokines (including inhibin, follistatin, and fibroblast growth factors) and their receptors, which are altered with differentiation.

The following sections will examine ES differentiation into endoderm, mesoderm and ectodermal derivatives, most typically following differentiation via EB.

### Endoderm

At about embryonic day 4 in the mouse embryo, primitive endoderm begins to form at the base of the ICM. Parietal endoderm and visceral endoderm then differentiate, and parietal endoderm migrates to line the yolk sac cavity. At

gastrulation, the visceral endoderm (hypoblast) is gradually replaced by embryonic endoderm of epiblast origin. Consistent with its early appearance in the embryo, endoderm forms when LIF is removed from cultures of ES, and is present on the outer surface of EB, where the pattern of endoderm specific gene expression is similar to that in the developing embryo.[1] Because of this, cystic EB are often used as models of yolk sac differentiation, and a number of agents have been reported to increase endoderm differentiation in EB.

Genes of the hepatocyte nuclear factor (HNF) family (e.g., HNF4, which is expressed in endoderm as soon as that layer can be identified) are expressed during endoderm lineage differentiation in EB. HNF4 is critical not in determination of the endoderm lineage, but in its later differentiation, while overexpression of HNF3 and 4 in ES induces genes typical of early (but not late) endoderm.[28] In general, genes thought to specify endoderm that have been examined to date appear to play a role in later differentiation rather than in early endoderm determination.

Interestingly, there is no endoderm in EB formed from ES null for the GATA-4 gene (a zinc finger—containing transcription factor expressed in primitive endoderm), but these cells are able to form endoderm in the presence of inducing factors—retinoic acid exposure produces visceral endoderm, whereas RA and db-cAMP results in formation of parietal endoderm.[6] In addition to being a very useful system to examine genes differentially expressed in these populations, the parent endoderm-deficient cell population (GATA-4 −/ −) provides the opportunity to study the role of endoderm-produced factors that increasingly appear to play a role in patterning of the early mouse embryo.

### Mesoderm

At gastrulation, a new layer of mesoderm is interposed between the ectoderm (epiblast) and underlying visceral endoderm (hypoblast) of the embryo. Mesoderm has been reported to form in EB following exposure to activin A or BMP4,[22] or when ES were transfected to express a dominant

negative truncated TGF-β receptor to block signaling by the TGF-β superfamily.[20] Consistent with this pattern, markers of mesoderm, particularly the T gene (*brachyury*), are expressed very early in the differentiation of EB. It is crucial in studies of this type to determine whether expression of an early lineage marker gene such as T is indicative of the formation of primitive mesoderm, or rather a transient state beginning a differentiation pathway.

Mesodermal differentiation, particularly into cells of the hematopoietic and muscle lineages, has been studied extensively in ES.

## Hematopoietic Differentiation

Perhaps the most intensely studied derivatives of EB are cells of the hematopoietic lineage.[51] After as little as 4 days in vitro, EB display foci of hematopoiesis; after 14 days in vitro, approximately 30% of EB develop endothelium-lined channels containing erythroblasts that resemble blood islands of the embryonic yolk sac.[13] Hematopoietic differentiation of ES has been employed extensively to examine the role of putative hematopoietic growth and differentiation factors including kit ligand, VEGF, EPO, IL-3, IL-6, IL-11, Steel factor, MCSF, and BMP4. Differentiation into hematopoietic lineages is typically carried out by aggregation (EB formation) followed by dissociation and replating in semisolid methylcellulose. Alternatively, ES have been grown on, or in medium conditioned by bone marrow stromal cells. The pattern of globin gene expression as well as the appearance of the various lineages appears to parallel that seen in vivo, with the ordered differentiation of erythroid cells, macrophages, neutrophils, mast cells, and megakaryocytes followed later and less efficiently by lymphocytes (e.g., Keller[24]).

Gene deletion studies in embryos and homozygous null ES have indicated a role for a number of genes in differentiation of hematopoietic precursors. Deletion of the proto-oncogene *vav*, the Lim domain protein rbtn 2, or scl, a helix-loop-helix transcription factor all completely block hematopoiesis in vitro, suggesting that they play a pivotal role in the very early stages of hematopoietic differentia-

tion.[51] Deletion of the Shp-2 protein tyrosine phosphatase in ES significantly reduces development of erythroid progenitors and completely blocks granulocyte–monocyte and mast cell precursor development.[38] Differentiation of GATA2 null ES indicates that hematopoiesis is blocked at the very early stem cell level, while the GATA1 gene appears to be required for survival of late committed erythrocyte precursors rather than for the early development of the definitive erythroblast lineage. Hematopoiesis in GATA1-null ES can be restored by expression of either mouse or chick GATA1 binding proteins, or by GATA3 or GATA4,[51] illustrating the important role of ES in examining the function of overlapping genes, and in studying structure/function relationships. The interesting observation that BMP4 is able to increase hematopoietic potential of EB[22] can be understood by recent evidence that GATA1 is downstream of BMP4 in a signaling cascade.

Somewhat surprisingly, only lymphocyte populations have been reconstituted following transplantation of ES derived hematopoietic stem cells into either Rag2- deficient mice (which contain no B or T lymphocytes[39]) or nude mice. These EB transplants may have failed to achieve long-term colonization because the hematopoietic precursor population could not complete their final maturation in vivo, or alternatively that these cells exhibit limited self renewal, i.e., they are not true stem cells. Recently, transplantation of hematopoietic precursors derived by treatment of ES with stromal cell-conditioned medium supplemented with IL-3- and IL-6-produced hematopoietic stem cells that repopulated lymphoid, myeloid and erythroid populations of irradiated mice.[36] Secondary engraftment was also successful, suggesting that a true stem cell may be isolated in this system.

Macrophages have been reported to be strikingly enriched in two step cultures exposed to IL-3 and MCSF.[29]

Vasculogenesis has also been studied in EB, as hematopoietic cells are present in regions lined with primitive endothelium, begging the question of the presence of a common precursor (hemangioblast), or alternatively, the requirement of an intact endothelium for erythropoiesis. When EB are trans-

ferred to the peritoneal cavity, a capillary plexus rapidly forms around them; when placed on the quail chorioallantoic membrane, a strong angiogenic response was observed,[40] suggesting that EB can model both processes.

## Muscle

The presence of foci of spontaneously contracting muscle fibers after 8–20 days in vitro is one of the most striking aspects of EB differentiation. These cultures are typically initiated in suspension, and then resulting embryoid bodies are plated onto adhesive substrates to achieve final differentiation. Expression of muscle specific structural genes (including myosin light and heavy chain genes, muscle specific actins, desmin, connexins, as well as muscle determination genes myf5, myogenin, myf6, MyoD1, and MRF4) proceeds in a pattern roughly paralleling that seen in the early embryo. The derived muscle cells form sarcomeres, have organized myofibrils, intercalated discs and functional gap junctions,[52] as well as functional $Ca^{2+}$ channels and receptors. TGF-β and RA treatment of ES cells has been reported to increase the number of cardiac and skeletal muscle cell derivatives, possibly due to a non-specific increase in mesoderm cell number. Similarly, many gene deletions which alter mesodermal patterning necessarily affect muscle cell differentiation.

Cardiomyocyte differentiation precedes differentiation of skeletal and smooth muscle cell types in embryoid bodies, as in the embryo. Specific lineages of sinus node-, atrium- and ventricle-like cells in cultures treated with RA have been identified using both patch clamping and selective gene expression.[55]

EB differentiation has indicated that the GATA4 transcription factor initially thought to be involved in regulation of cardiomyocyte differentiation is non-essential for myocyte differentiation,[32] while deletion of the Cripto-1 gene (a growth factor expressed in the heart) resulted in EB totally lacking cardiac myocytes, although other cell types including skeletal muscle cells were present. Re-expression of the Cripto-1 gene rescued the development of cardiomyocytes, suggesting it plays an essential role in their differen-

tiation.[56] Deletion of the merosin (laminin2)-specific alpha chain in ES resulted in the formation of very unstable myotubes with collapse and degeneration of EB-derived myocytes, similar to the pattern seen in the severe form of muscular dystrophy.[26]

In an interesting study, ES cells were transfected with an expression construct in which aminoglycoside phosphotransferase gene expression was driven by the cardiac myosin heavy chain promoter.[25] ES were differentiated as EB followed by plating and selection in high antibiotic concentrations, producing a surviving population in which >99% were cardiomyocytes. When these cells were injected into adult heart, they formed stable grafts as long as 7 months, the latest timepoint examined.

Differentiation of other muscle cell types from ES has not received as much attention as cardiomyocytes, although vascular smooth muscle formed when EB were exposed to RA and db-cAMP on days 7–11 of differentiation as EB.[14] Expression of the bHLH muscle regulatory gene MyoD followed by EB formation and treatment with DMSO has been reported to result in nearly pure populations of skeletal muscle.[12] However in another study, constitutive expression of MyoD1 and differentiation as EB resulted in only partial differentiation of ES to skeletal muscle cells, even though all cells expressed MyoD1 mRNA. These studies highlight the difference in differentiation via EB in defined medium (+ DMSO) in contrast to differentiation in serum-containing medium–conversion to skeletal muscle was high in the former and incomplete in the latter, despite expression of the same gene! As Shani et al.[47] note, "environmental factors (present in EB themselves and in serum) control the expression of its myogenic differentiation function."

Gene deletion and differentiation of ES have produced some surprising results—e.g., deletion of the myogenic regulatory gene myf-5 did not alter the myogenic potential of the ES,[8] while constitutive expression of the M-twist bHLH gene delayed and reduced the number of skeletal muscle cells in EB.[42]

## Additional Mesodermal Derivatives

Chondrocytes[13] have been observed in sectioned EB. Adipocytes form in approximately 60% of EB exposed to $10^{-8}$ M RA on days 2–5 of EB culture; addition of insulin and T3 to the medium significantly increases the number of adipocytes present.[11]

## Ectodermal Derivatives

Because of their origin from the inner cell mass/early epiblast, it has been stated that ES most resemble ectodermal tissue; and in fact, ES cells do express many structural genes (intermediate filament proteins, extracellular matrix proteins, cell adhesion molecules) expressed by primitive ectoderm. Since exposure of ES to activin A, or BMP4, or expression of a truncated TGF-$\beta$ receptor construct in ES, results in differentiation to a mesodermal phenotype, follistatin, chordin or noggin treatment of these cells might be expected to produce an ectodermal or neuroectodermal phenotype. In fact, ES grown in defined medium without added factors differentiate into cells that express Pax 6, a marker of early neuroepithelium,[54] and expression of a noggin cDNA in ES results in robust neuronal differentiation[34] (Fig 3F). Despite the obvious use in grafting, little effort has been made to date to examine the ability of ES to differentiate into epidermis. Most work in this lineage has been devoted to neuronal (and glial) differentiation of ES, and most has relied on differentiation of ES into EB and induction by retinoic acid.

## Neurons and Glia

Protocols employed to achieve neuronal differentiation rely largely on the formation of EB in complete medium (15–20% serum) in the presence of retinoic acid, followed by dissociation and plating on tissue culture plastic.[4] Differentiation has been followed by examining morphology, the expression of neuron specific markers [including neurofilament proteins (Fig. 3D,E), the dendrite specific marker MAP 2, Tau, neurocan, neural tubulin, Wnt 1, GAP 43, MASH1, and neurotransmitter genes], and determination of whether cells are electrically excit-

able when examined using patch-clamp. Glial differentiation has been monitored by the expression of the glial fibrillary acidic protein, OP4 (for astrocytes), and GalC and O4 (for oligodendroglia). The intermediate filament protein nestin and Pax6 have been employed to mark primitive neuroepithelial cells. Since EB differentiation progresses in the presence of non-specific differentiating agents (typically RA) in the presence of serum, with waves of successively differentiating cells secreting multiple growth factors, it is not surprising that this paradigm produces mixed populations of neuronal and glial cells. For some applications, this may actually be an advantage, as glial cells secrete survival factors for the neurons, and also form an adhesive substratum for the neuronal cells in vitro. In other cases, it is irrelevant. If the goal is, for example, to examine the role of membrane pumps following gene deletion, then only a few differentiated neurons are required to determine if membrane characteristics have been altered.

Research into the role of growth and survival factors for ES-derived neurons is beginning. Differentiation of ES in defined medium without additional growth factors appears to produce cells that express genes typical of early neuroepithelium,[54] and it has been reported that NGF increases the number of neuron like cells in EB. Removal of the mitogen bFGF from cultures of neurally differentiating ES produces a population of neuroepithelial stem cells which are over 80% nestin positive.[33] Interestingly, based on their gene expression profiles (e.g., Hoxa7, Otx1, En1) these cells appear to belong to a CNS rather than PNS class. When EB are cultured in the presence of CNTF, an astrocyte phenotype is induced, while T3 promotes oligodendroglial differentiation. Interestingly, when these glial cells were transplanted into the ventricle of myelin deficient (md) rat embryos, they engrafted widely and formed myelin sheaths around host axons.[9]

To date, the focus of much of this work has been on achieving neuronal differentiation, with little emphasis on examining the role of regulatory genes in this process. However, constitutive expression of the bHLH neuronal de-

termination genes NeuroD2 and NeuroD3 in ES (in defined medium) produces neurons with distinct morphologies.[35] Although there have been few studies examining the ability of gene-targeted ES to differentiate into neurons and glial cells, one study examined the role of gangliosides (previously thought to be essential in neuronal differentiation) by disrupting the GD3 synthase gene in ES. Surprisingly, neurogenesis was not impaired, suggesting a non-essential role for GD3 in this process.[23]

When serum was reduced to 1%, or when induced with RA, >90% of cells in EB were reported to express NF protein.[18] Neural differentiation was concentration dependent, with neural differentiation peaking at 100 nM RA; cardiac muscle formed without RA, results which are highly reminiscent of McBurney's original work with EC cells. Dinsmore has reported that when EB induced in 500 nM RA for 4 days, then dissociated and plated in semidefined medium (serum free, but containing "pituitary extract"), greater than 90% of the resulting cells were neuronal, and that neurons transplanted to lesioned rat neostriatum survived for periods of 6 weeks. Unlike undifferentiated ES that continued to proliferate in the striatum, and had few differentiated neurons within the mass, neuronal cells appeared to incorporate into the brain, although the extent of neurite outgrowth could not be assessed in these preparations.[12] Certainly, use of ES for grafts such as this have considerable advantages over using fetal material: cells could be transfected to express region specific or neurotransmitter genes, anti-apoptotic genes, or anti-amyloid constructs. They could be co-injected with glial cells for supporting factors, and there is an unlimited source of starting material.

## FUTURE DIRECTIONS

Perhaps the most obvious success of ES differentiation has been the study of the developmental potential of the ES themselves, after gene deletion resulted in early embryonic lethality. Important new information has also been obtained regarding growth factor expression and roles in cell type-specific differentiation, and new information regarding the role of secreted

signaling factors in lineage decisions is developing. Although these studies have produced otherwise unobtainable new data, most have used the unspecific method of differentiation as EB in serum-containing medium, and not surprisingly have resulted in differentiation of multiple cell types. Clearly, as more is known about lineage specification genes and defined medium/growth factor requirements of these cells, it will be possible to more precisely control cell type specification. In addition to monitoring phenotype and gene expression patterns, these studies will ultimately require careful assessment of the *behavior* of differentiated derivatives of ES by reintroducing them into embryos—either in whole embryo culture or via exo utero surgery.

Somewhat surprisingly, given their

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## Clearly, as more is known about lineage specification genes and defined medium/growth factor requirements of these cells, it will be possible to more precisely control cell type specification.

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similarity to the early embryo, few toxicology or teratology studies have been carried out using ES, although some EC lines have been employed to study the mechanism of action of toxicants. Rarely, too, have ES been employed as primary models of disease or disease process (however, see Kuang et al.[26]).

Finally, since it is unlikely that fetal donors will provide sufficient cells for transplantation and tissue engineering needs, there is great potential for the recently derived pluripotent human cell lines in these intervention strategies. Given that both the mouse and human genome projects will soon be complete, the ability to compare and test the expression and role of newly identified genes using rapid, ES-based differentiation screens (e.g.,

Evans et al.[15]), and the ability to examine gene expression during the very early differentiation of human cells—a stage of development not usually accessible for investigation—will provide unsurpassed insight regarding this critical stage of development.

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# The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium

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## SUMMARY

The *in vitro* developmental potential of mouse blastocyst-derived embryonic stem cell lines has been investigated. From 3 to 8 days of suspension culture the cells form complex embryoid bodies with endoderm, basal lamina, mesoderm and ectoderm. Many are morphologically similar to embryos of the 6- to 8-day egg-cylinder stage. From 8 to 10 days of culture about half of the embryoid bodies expand into large cystic structures containing alphafoetoprotein and transferrin, thus being analagous to the visceral yolk sac of the postimplantation embryo. Approximately one third of the cystic embryoid bodies develop myocardium and when cultured in the presence of human cord serum, 30 % develop blood islands, thereby exhibiting a high level of organized development at a very high frequency. Furthermore, most embryonic stem cell lines observed exhibit similar characteristics. The *in vitro* developmental potential of embryonic stem cell lines and the consistency with which the cells express this potential are presented as aspects which open up new approaches to the investigation of embryogenesis.

## INTRODUCTION

Blastocyst-derived embryonic stem (ES) cells are established *in vitro* from substrate-attached blastocysts without passage of the cells through tumours. They are maintained in an undifferentiated pluripotent state by culturing on an embryonic fibroblast feeder layer and spontaneously differentiate in the absence of feeder layer cells. Several methods have been applied successfully to the establishment of ES cell populations. Evans & Kaufman (1981) produced lines of pluripotent cells from the epiblast of delayed-implantation blastocysts. At the same time Martin (1981) established cell lines from cultures of immunosurgically isolated inner cell mass cells which were grown in medium conditioned by teratocarcinoma-derived embryonal carcinoma (EC) cells. It is now known that ES cell lines can be established without delayed-implantation blastocysts or EC-cell-conditioned medium (Robertson, Evans & Kaufman, 1983; Axelrod, 1984). There is no indication that there are significant differences between the cell populations derived in these ways.

*Key words:* ES cells, visceral yolk sac, blood islands, myocardium, mouse embryo.

Teratocarcinoma-derived EC cells are produced from a limited number of mouse strains which have the capacity to develop teratocarcinomas and have been used as an *in vitro* model system for embryonic development (Solter & Damjanov, 1979; Martin, 1980). The *in vitro* differentiation potentiality of EC cells has been studied either with the help of chemical inducers (Jones-Villeneuve, McBurney, Rogers & Kalnins, 1982; McBurney, Jones-Villeneuve, Edwards & Anderson, 1982; Paulin *et al.* 1982) or with cell lines which spontaneously differentiate under varying culture conditions (Rosenthal, Wishnow & Sato, 1970; Martin & Evans, 1975*a, b*; Sherman & Miller, 1978; Darmon, Bottenstein & Sato, 1981; Pfeiffer *et al.* 1981; Rizzino, 1983). Few EC cell lines are capable of spontaneous differentiation, and of these very few have the capacity to form cystic structures with phenotypic similarities to the postimplantation embryo (Rosenthal *et al.* 1970; Martin, Wiley & Damjanov, 1977; Cudennec & Nicolas, 1977).

The cells of blastocyst-derived ES cell lines may be quite similar to normal embryonic cells and in most cases are probably less altered by their *in vitro* environment than are the cells of most teratocarcinoma-derived cell lines by a tumour environment. This is most clearly evidenced by the remarkably high frequency with which ES cells can be used in blastocyst injection experiments to form chimaeras of a broad tissue spectrum as well as germ-line chimaeras (Bradley, Evans, Kaufman & Robertson, 1984). Other advantages of ES cells lie in the fact that they can be made from mouse strains which carry recessive lethal mutations (Magnuson, Epstein, Silver & Martin, 1982) or from parthenogenic embryos (Robertson *et al.* 1983). The degree, however, to which the use of ES cell lines will be beneficial in investigating the lesions occurring in such strains will be largely dependent upon the degree to which the lesion-bearing lines and the non-lesion-bearing lines of the same genetic background will be able to provide some semblance of organized *in vitro* development. It is therefore necessary to know the developmental potential of these cells in order that the fullest possible range of questions can be directed within the boundaries of this potential.

All blastocyst-derived ES cell lines so far described spontaneously differentiate and form cystic embryoid bodies (Evans & Kaufman, 1981; Martin, 1981; Robertson *et al.* 1983). The degree to which organized development similar to that of the embryo occurs within them, however, has not been described. The investigation reported here has done this by analysing the most advanced embryonic-like structures developed by a blastocyst-derived cell line, ES-D3. It has compared the extent of this development, as well as that of several other ES cell lines from 129 and C57 mouse strains, to the postimplantation embryo. It is shown that the blastocyst-derived cells can differentiate at a remarkably high frequency to form heart and blood cell-containing cystic structures similar to the visceral yolk sac of the embryo. A close analysis is made of the fluid content of the cystic structures, the erythrocytes of the blood islands and the morphology of the heart-like structures. The unique advantages which ES cells may provide to the study of embryonic development are outlined.

## MATERIALS AND METHODS

### Establishment and maintenance of cell lines

The ES cell line ES-D3 was derived from eight 129/Sv +/+ 4-day blastocysts, day of plug detection being set at 1 day of embryonic development. The ES-D3/4 and ES-D3/7, and ES-D3/10/5 cells are first- and second-order colony subclones, respectively, of the ES-D3 cells. ES-632 cells are a single-cell subclone similarly established from C57BL/6 blastocysts. After approximately 1–2 days of culture on a feeder layer of BALB/c 16- to 18-day embryonic fibro-blasts (generously provided by Dr U. Koszinowski, Bundesforschungsanstalt für Viruskrank-heiten der Tiére, Tübingen, W. Germany, see also Wobus, Holshausen, Jäkel & Schöneich, 1984) in Nunclon Delta SI tissue culture dishes, the inner cell mass cells were picked out, mechanically dissociated by gentle pipetting and transferred to a new feeder layer. Embryonal medium, consisting of 10 % foetal calf serum, 10 % newborn calf serum and 0·1 mm-$\beta$-mercapto-ethanol in DMEM (Robertson *et al.* 1983), was changed every 2 days and the ES cells were transferred to new feeder layers about twice weekly. Embryonal medium was used during the establishment of all ES cell lines. After the lines were stable, they were maintained in the undifferentiated state on feeder layer cells. The maintenance medium was 15 % foetal calf serum in DMEM to which $\beta$-mercaptoethanol was added to 0·1 mm. The feeder layers were produced by treating the embryonic fibroblasts with 10 $\mu$g.ml$^{-1}$ mitomycin-C for 3·5 h. The feeder layer cells were plated at approximately 5 × 10$^6$ cells per 90 mm tissue culture dish.

### Cell culture under differentiation conditions

All ES and EC cells were cultured in the absence of embryonic fibroblasts in standard medium (15 % foetal calf serum in DMEM) either in tissue culture dishes, or in suspension in bacterial dishes or bottles placed on a rotary shaker. Cells were cultured in tissue culture dishes either under monolayer conditions using Falcon or hydrophilic petriperm (Heraeus) dishes at approximately 2 × 10$^5$ cells.ml$^{-1}$ or under micromass culture conditions in which 10 $\mu$l droplets of 20 000 cells each were added to 24-well tissue-culture dishes (Costar) or to hydrophilic petriperm dishes. After allowing the cells to attach for 4 h the dishes were gently flooded with medium. Suspension culture in bacterial dishes (Greiner) also contained 2 × 10$^5$ cells.ml$^{-1}$. Suspension culture on a rotary shaker was performed at 70 r.p.m. with 20 mm-Hepes-buffered standard medium with approximately 10$^5$ cells.ml$^{-1}$. No significant differences could be found in the developmental potentiality of ES cells between the two types of suspension culture, or between monolayer and micromass cultures. 'Days of culture' will refer to the days of culture after switching the cells to differentiation conditions.

### Karyotype

Chromosome spreads were performed as described (Triman, Davisson & Roderick, 1975) using the modifications kindly provided by S. Adolph (Klinische Genetik der Universität Ulm, W. Germany). Before spreading onto microscope slides pretreated with ethanol/ether (1:1), cells were treated 1–2 h with 10 $\mu$g.ml$^{-1}$ colcemid, 10–15 min with 0·56 % KCl and 10, 20 and 39 min successively with methanol/acetic acid (3:1) at 4 °C. G-banding was done as described (Seabright, 1971).

### Histology and immunofluorescence

Indirect immunofluorescence tests with the monoclonal antibody against trophectodermal cytokeratin-like filaments TROMA-1 (Brûlet, Babinet, Kemler & Jacob, 1980) and benzidine staining of erythrocytes in blood islands were performed on methanol-fixed (−20 °C, 10 min) cryostat sections of cystic embryoid bodies. The anti-mouse macrophage monoclonal antibody (MAS 034, Sera-Lab) was used in indirect immunofluorescence tests on the easily dissociable cells from mechanically disrupted cystic embryoid bodies. After pipetting the cystic embryoid bodies in and out of a Pasteur pipette several times, the single cells were centrifuged onto a gelatine-

coated microscope slide using a cytocentrifuge (Cytospin 2, Shandon) and fixed with 4 % para-formaldehyde (4 °C, 10 min).

## Immunoprecipitation and gel electrophoresis

Immunoprecipitation and electrophoresis of the fluid content of the *in vitro* cystic embryoid bodies was done by using the *Staphylococcus aureus* procedure of Kessler (1975) as applied by Vestweber & Kemler (1984). Cystic embryoid bodies were incubated in methionine-poor standard medium in the presence of [$^{35}$S]methionine (50 $\mu$Ci.ml$^{-1}$) for 18 h after which the cavity fluid was collected either with a small Hamilton syringe or by gently breaking the cavities open and washing out the contents. Alphafoetoprotein (AFP) antiserum and affinity-purified anti-transferrin were kindly provided by E. Adamson (La Jolla Cancer Research Foundation, California) and were used at 20 $\mu$g.ml$^{-1}$ (IgG fraction) and 3 $\mu$g.ml$^{-1}$, respectively, in the immunoprecipitations.

Blood cells for isoelectric focusing of haemoglobins were isolated and treated according to a modification of the method described by Cudennec, Delouvee & Thiery (1979). Briefly, adult 129/Sv blood cells were washed once in PBS, resuspended in 0·25 M-sucrose (containing 1 % (v/w) trasylol (Sigma) and 0·1 % KCN), centrifuged, and the cell pellet lyophilysed. Entire day-11 visceral yolk sacs (129/Sv) and blood-island-containing ES-D3 *in vitro* cystic embryoid bodies were ruptured and rinsed several times in PBS before resuspension in the above buffer. The 0·12 mm-thick isoelectric focusing gels were prepared and run on a flat-bed gel apparatus (LKB-Ultrophor) as described (LKB technical bulletin, modified by Dr Peter Symmons, Max-Planck-Institute for Developmental Biology, Tübingen, W. Germany) using pH 7–9 ampholines (Serva).

## Electron microscopy

Samples were fixed for 2 h in 2·5 % glutaraldehyde in PBS, postfixed 1 h each in 1 % osmium tetroxide in PBS and 2 % uranyl acetate in 70 % alcohol, embedded in Epon, sectioned, contrasted 8 min at 25 °C in lead citrate in an LKB 2168 Ultrostainer and viewed with a Siemens Elmiskop 102 electron microscope at 80 kV.

## RESULTS

### Establishment and maintenance of the embryonic stem cells

After approximately 1–2 days of culturing 129/Sv +/+ blastocysts on a mitomycin-C-treated embryonic fibroblast feeder layer, clumps of inner cell mass cells from eight blastocysts (Fig. 1A) were picked out, mechanically dissociated by pipetting and transferred to a new feeder layer. In our hands 129/Sv ES cell lines could be established from approximately 5 % of the attached blastocysts and C57BL/6 cell lines from about 10 %. As long as the cells were maintained on a dense layer of feeder cells and replated every two days on a new feeder layer, differentiation was inhibited. This was determined morphologically (Fig. 1B) and by immunofluorescence with antibody markers specific for undifferentiated and differentiated embryonic cells (Kemler *et al.* 1981; Kemler, 1981; not shown). When carefully maintained in culture in the undifferentiated state as described above, ES cell lines can be kept in culture from 3 months to a year and can be freeze thawed several times without any apparent loss in developmental potential.

If the cells were grown on tissue culture plates without a feeder layer, differentiating, substrate-attached cells began growing out from the undifferentiated cell
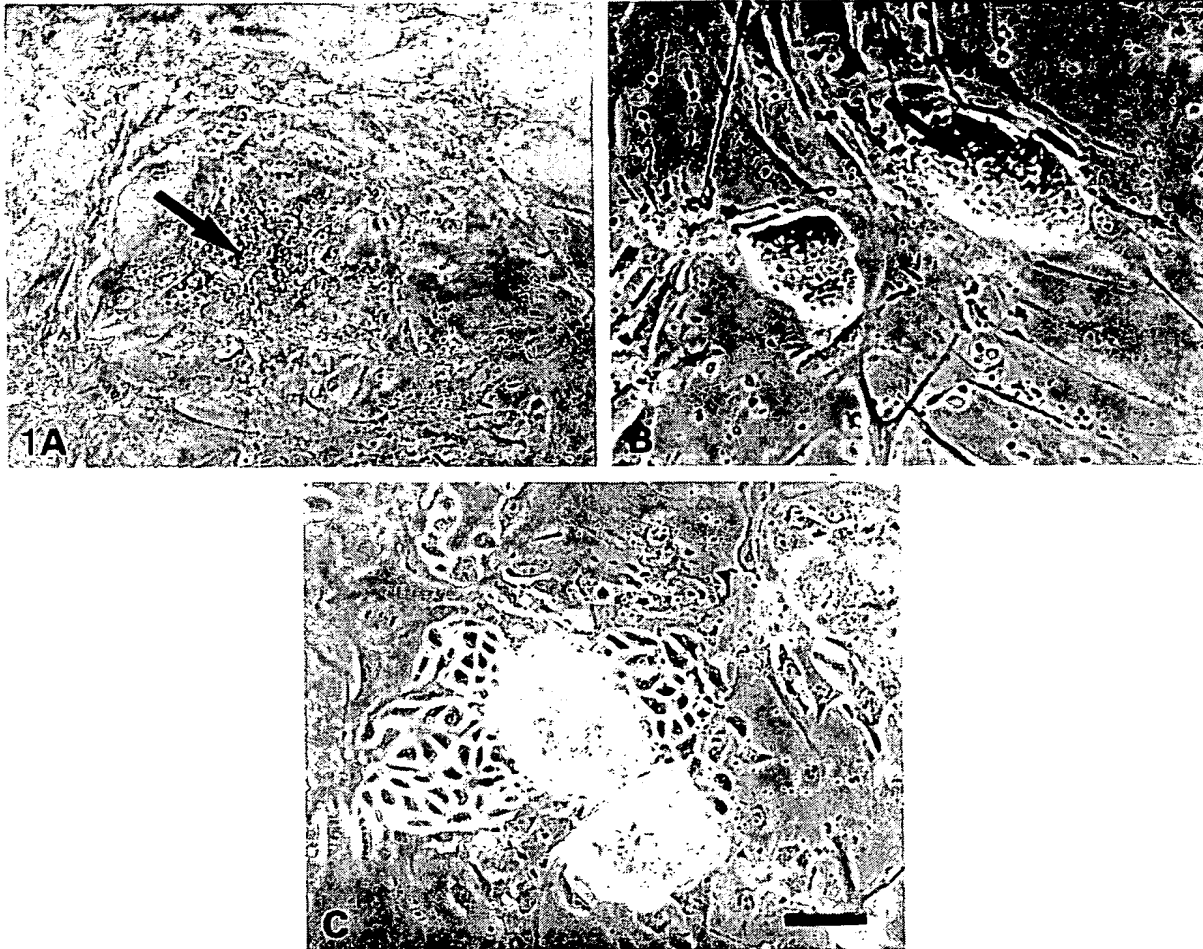
Fig. 1. ES cells during the establishment of cell lines, during maintenance in the undifferentiated state, and under differentiation conditions. 4-day 129/Sv blastocysts were allowed to attach to an embryonic fibroblast feeder layer. (A) Attached blastocysts when the inner cell mass cells (arrow) are removed and transferred to a new feeder layer after 2 days of culture. (B) Clumps of undifferentiated cells (arrow) being maintained on a feeder layer. (C) Differentiating ES-D3 cells after 2 days of culture on a gelatin-coated tissue culture dish in the absence of feeder layer. (Gelatine treatment is not necessary for ES cell differentiation.) Note the flat, differentiated cells growing out from the stem cell clumps. The few feeder layer cells remaining after transfer of ES cells to feeder-layer-free dishes are not sufficient to prevent differentiation. A–C: Bar = 100 μm.

clumps within 24 h (Fig. 1C). In all experiments the various ES cell lines seemed to behave identically. The blastocyst-derived cell lines have normal diploid karyotypes. Forty chromosomes were found in 62 % and 45 % of the cells, respectively, and both cell lines were XY. The G-banding pattern from one cell was analysed and revealed no translocations or metacentric chromosomes (Fig. 2).
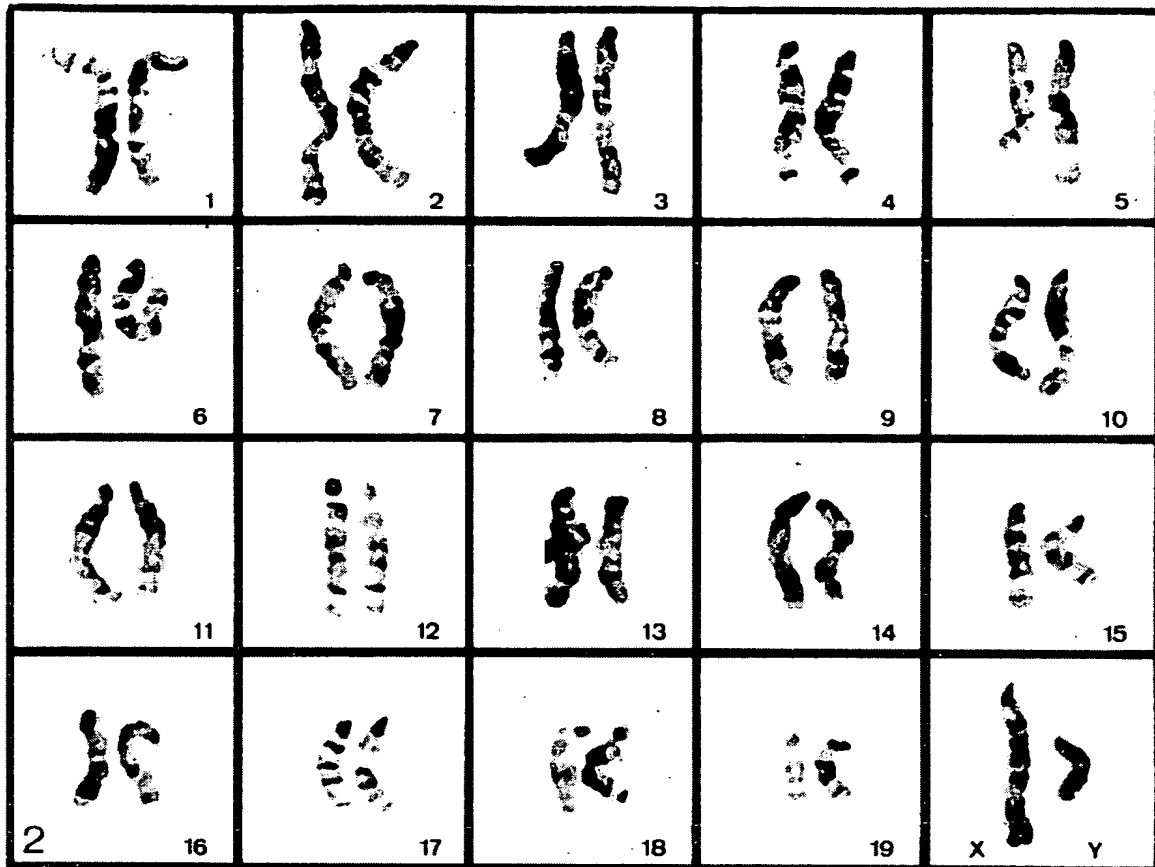
Fig. 2. Karyotype of a typical ES-D3/10/5 cell which has a normal diploid set of 40 chromosomes, has an XY constitution and contains no detectable abnormalities. ES-D3 cells: Out of 31 metaphase cells 62 % had 40, 25 % less than 40 and 13 % more than 40 chromosomes. ES-D3/10/5 cells: Out of 49 metaphase cells 45 % had 40, 32 % less than 40 and 22 % more than 40 chromosomes.

In vivo *formation of tumours*

To demonstrate that the ES cells could differentiate into products of all three germ layers, they were injected either subcutaneously or intraperitoneally into syngeneic mice, and the resulting tumours were analysed. When injected subcutaneously, solid teratocarcinomas were formed which contained large vacuoles enclosed by ciliated epithelial cells (preliminary evidence suggesting that these vacuoles have similarities to the brain ventricles), cross-striated muscle, cartilage, calcified cartilage, melanocytes and keratin sworls (not shown). When injected intraperitoneally, a mixture of mesenterically adherent solid tumours and un-attached cystic embryoid bodies were formed. The latter contained outer and inner epithelial layers with areas of mesoderm in-between. The mesodermal areas

contained blood islands with embryonic haemoglobin-containing erythrocytes (see Fig. 8B for example) and contracting embryonic heart muscle cells (not shown). Immunofluorescence tests on cryostat sections of the cysts (not shown) showed that the cystic cavities contained large quantities of AFP.

## In vitro *cultures*

In all of the experiments described below differentiating cells were cultured in standard medium without the addition of factors or inducers of any kind. Regardless of the type of culture (suspension (bacterial dishes or rotary shaker) or monolayer) most of the cells formed aggregates. If the aggregates were allowed to attach to the substrate (or remained attached), cells proliferated out from them along the substrate and differentiated into a wide variety of structures morphologically
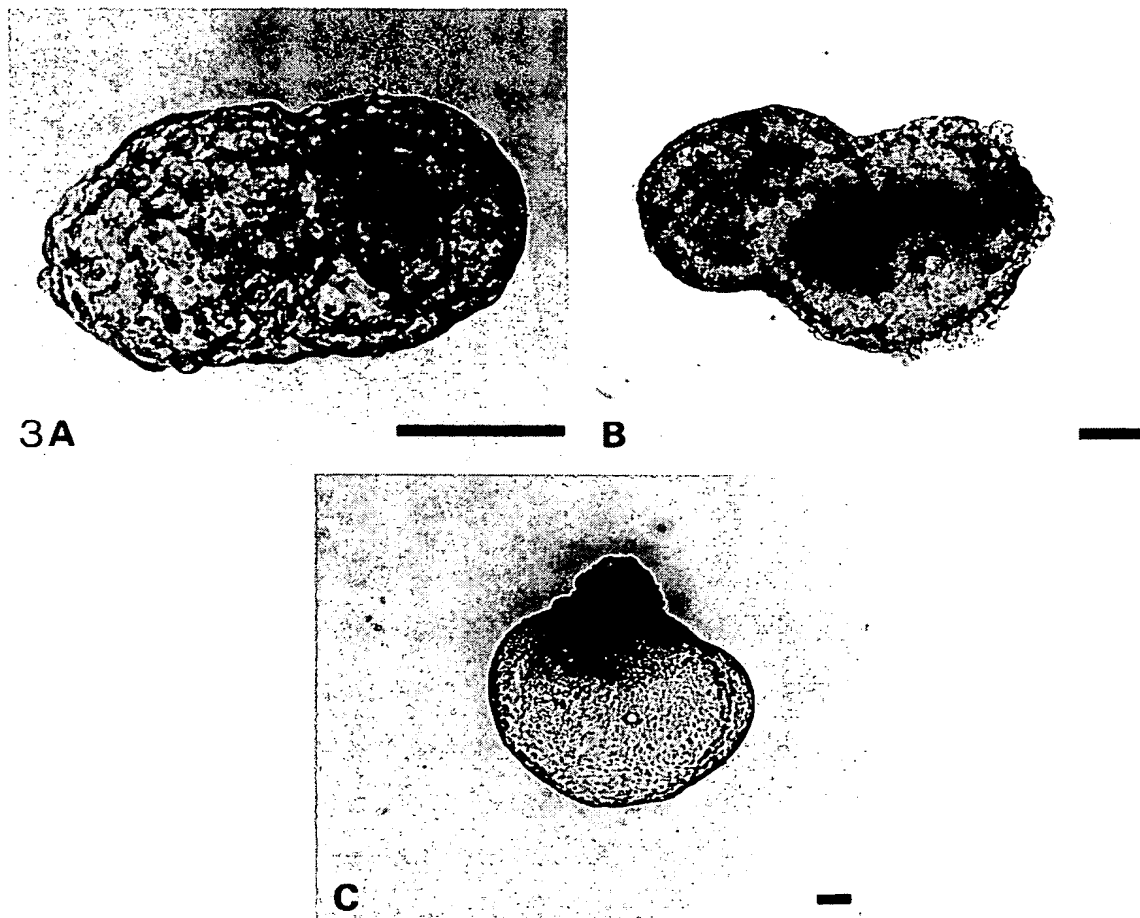


Fig. 3. Embryoid body development. ES-D3 cells were cultured under differentiation conditions in bacterial dishes for 4 days (A), 8 days (B) or 11 days (C). Bars = 100 $\mu$m.

identified at the light or electron microscopic levels as glandular, heart (see Fig. 5, for example), skeletal and smooth muscle, cartilage, nerve cells, keratin sworls, melanocytes and embryoid bodies (not shown). If, however, the aggregates were maintained in suspension, they developed only into embryoid bodies.

After 5 days of culture about 60 % of the embryoid bodies had developed into structures with an outer layer of endoderm bordered by a basal lamina within which the inner cells had condensed into a layer of columnar ectoderm-like cells. Many of these complex embryoid bodies were found to be polarized into two parts and appeared to be similar to the egg-cylinder stage of the 5-day embryo (Fig. 3A). Whether the two portions correspond to the extraembryonic and embryonic parts of the egg-cylinder-stage embryo is unclear. The markers we employed do not clearly distinguish between embryonic visceral on the one hand and extraembryonic visceral, primitive or parietal endoderm on the other. No trophoblast giant cells were ever seen during the entire culture period. During the next few days of culture there was a great deal of growth (compare Fig. 3A to 3B). By 8 days of culture an endodermal transition to the visceral type occurred along with the transition from complex to cystic embryoid bodies. After approximately 11 days of culture, many cystic structures were present (Fig. 3C) which looked similar to 8- to 10-day yolk sacs. After 3 weeks of culture, most developmental processes as well as growth had ceased, even though the cystic structures were viable for several more weeks.

## Identification of cystic embryoid body as visceral yolk sac

The production of AFP and transferrin is characteristic of visceral yolk sac endoderm (Dziadek & Adamson, 1978; Adamson, 1982). Consequently, the fluid content of [$^{35}$S] methionine-labelled cystic embryoid bodies was electrophoretically analysed for total content (Fig. 4, lane 2) and by immunoprecipitations with anti-AFP and anti-transferrin (Fig. 4, lanes 3 and 4; the respective immunoprecipitations from 12-day embryonic visceral yolk sac: lanes 5 and 6). The total protein composition of the cavities consisted predominantly of AFP and transferrin with a few minor proteins of approximately 25 000, 45 000 and 300 000 relative molecular mass ($M_r$), presumably apolipoproteins A-I, A-IV and B, respectively (see Shi & Heath, 1984; Meehan et al. 1984) the 300 000 $M_r$ protein which was precipitated by anti-transferrin was not precipitated from the labelling medium of embryonic visceral yolk sac cells (Fig. 4, lane 6). Electron micrographs of the outer endoderm cells of the cystic embryoid bodies (not shown) revealed many microvilli, electron-transparent cytoplasmic vesicles and junctional complexes as previously reported for EC cell cystic embryoid bodies (Martin et al. 1977), all of which are characteristic of visceral endoderm. Immunofluorescence tests on cryostat sections of cystic embryoid bodies using monoclonal antibodies TROMA 1 (positive for visceral and parietal endoderm, Kemler et al. 1981) and TROMA 3 (positive only for parietal endoderm, Boller & Kemler, 1983) (not shown) were consistent with the findings that the endodermal layer of the cystic bodies consisted predominantly of visceral yolk sac.
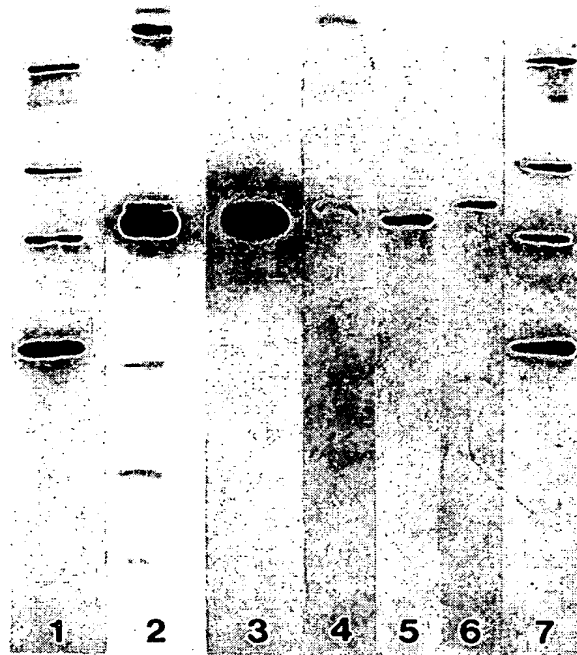
Fig. 4. SDS–PAGE and immunoprecipitation analysis of the protein content of ES cell cystic embryoid body cavities. Large, 2–4 mm in diameter ES-D3 or ES-D3/10/5 cystic embryoid bodies cultured for 2–4 weeks in bacterial or hydrophobic petriperm dishes (lanes 2–4), or freshly prepared 12-day visceral yolk sac (lanes 5, 6) were incubated with [$^{35}$S] methionine. Total labelled protein from cystic cavities (lane 2). Immunoprecipitation of cavity content (lanes 3,4) or labelled culture supernatant (lanes 5,6) with anti-AFP (lanes 3,5) or anti-transferrin (lanes 4,6). Note the high relative molecular mass protein coprecipitated by anti-transferrin from the cystic cavity contents but not from embryonic visceral yolk sac culture supernatant. Lanes 1, 7: protein relative molecular mass markers myosin heavy chain, 200 000; phosphorylase, 97 000; albumin, 68 000; and ovalbumin, 45 000.

*Myocardium*

After at least 8 days of suspension culture about one third of the ES cell cystic embryoid bodies began rhythmically contracting in areas where their surface was quite thick. Identically contracting structures could be found in micromass cultures and were analysed with respect to tissue organization and muscle type. Micrographs of video sequences of a highly organized beating structure (Fig. 5A, B) show the relaxed and contracted phase, respectively, of one contraction. The arch-shaped ridges (arrows) are phenotypically analogous to myocardium. An aggregate of cells was trapped in the endocardial-like cavity and moved back and forth during the contraction (arrowheads). Also associated with this structure were endothelial capillaries some of which also contained trapped cells which moved with the contractions (not shown). Electron micrographs showed that the muscle cells inside
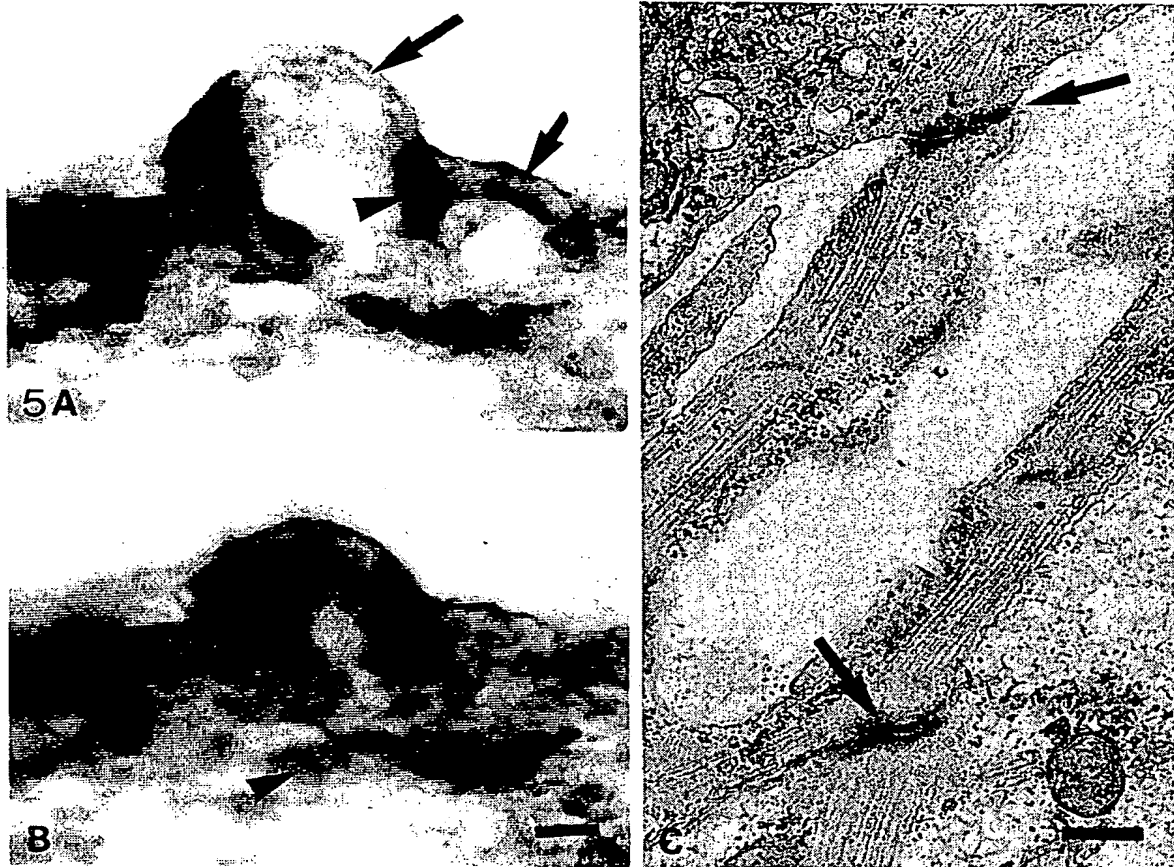
Fig. 5. Video micrographic and electron microscopic analysis of ES myocardial cells. (A,B). Polaroid photographs of video sequences of the relaxed and contracted phases, respectively, of one contraction. ES-D3 cells were grown in micromass culture. Myocard-like ridges (arrows) can be easily recognized in the relaxed phase of the contraction. Inside the endocard-like cavity a cell aggregate can be seen (arrowheads) which moves about 30 $\mu$m during the contraction. (C) Electron micrograph of ES-D3 cells which had been rhythmically contracting. The cells had been cultured for 4 days in suspension followed by 17 days on hydrophilic petriperm dishes. Note the intercalated disks which lie at the myofibrillar Z-bands of adjacent cells (arrows), structures characteristic of cardiac muscle cells. A,B: bar = 100 $\mu$m. C: bar = 0·5 $\mu$m.

Fig. 6. Dark-field photograph of ES cell cystic embryoid body. ES-D3/10/5 cells were cultured for 12 days on bacterial dishes. Besides the red blood islands, mesodermal thickenings (white areas) as well as endodermal subcompartmentalization can be seen. Bar = 200 $\mu$m.

Fig. 7. Histological and immunofluorescence analysis of ES cell cystic embryoid body blood islands. Cryostat sections of one blood-island-containing cystic embryoid ascites tumour removed 4 weeks after intraperitoneal injection of 5 × 10⁶ ES-D3/10/5 cells. (A) Benzidine stain. In the inner (top) and outer (bottom) endothelial cell layers, only the nuclei are stained. Between these layers the cytoplasm of the erythrocytes (arrow) are stained as well. (B) Indirect immunofluorescence with TROMA 1. Endothelial cells are positively stained and mesodermal cells, including erythrocytes, are unstained. A,B: Bar = 50 $\mu$m.
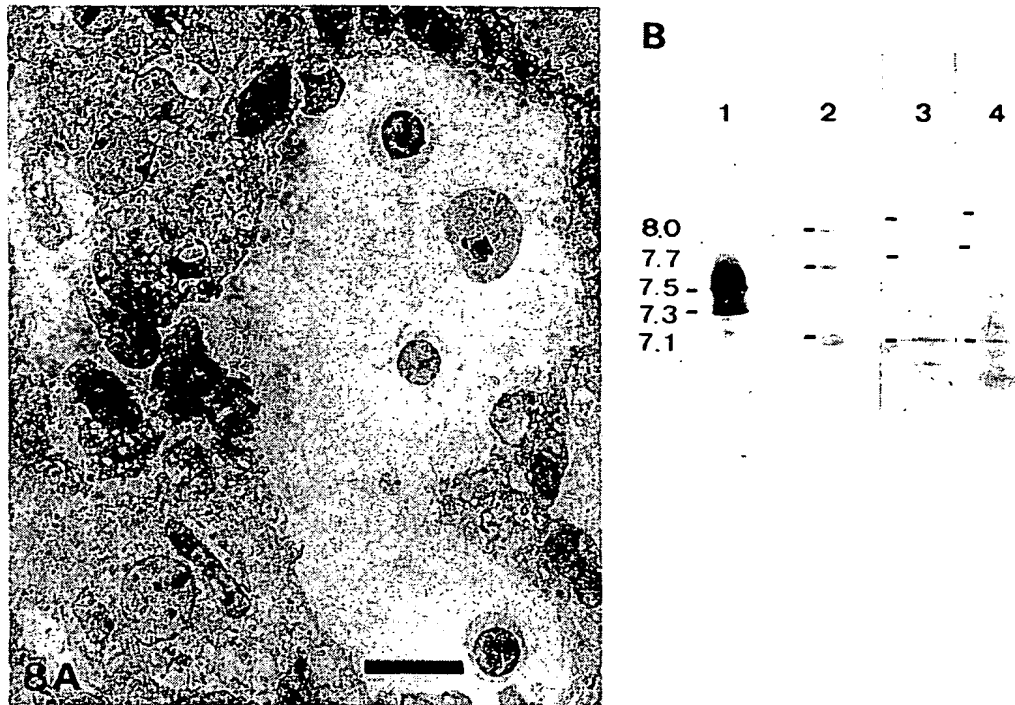
6



7a



7b
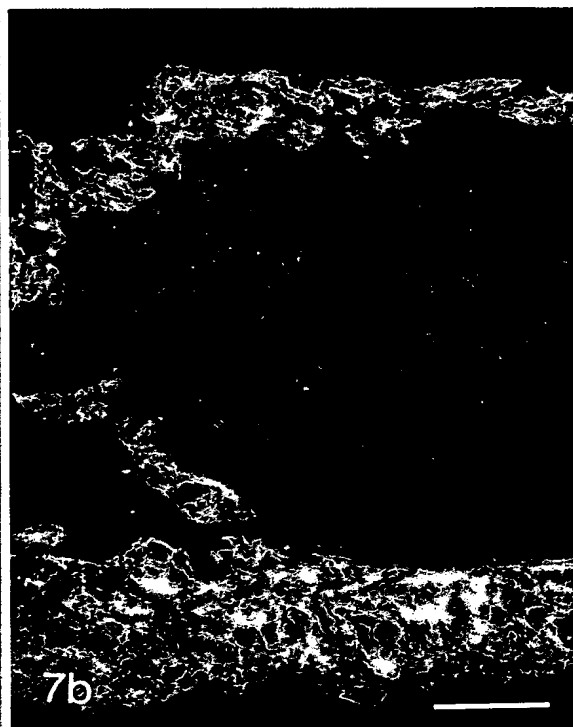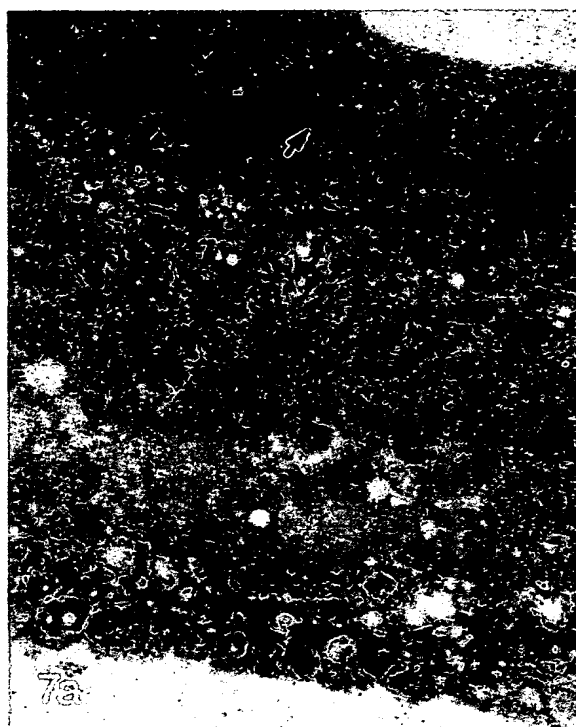
Fig. 8. Characterization of erythrocytes from ES cell cystic embryoid bodies and cystic tumours. (A) Electron micrograph of a blood island in an ES-D3/10/5 cystic embryoid body after 13 days of culture in a hydrophobic petriperm dish. The blood island consists of an endoderm-bordered cavity which contains unattached, nucleated erythrocytes typical of all yolk sac-derived red blood cells. (B) Isoelectric focusing of haemoglobin from three different gels (lanes 1 and 2, lane 3, and lane 4). The pH values indicated at the side are designated by a small line at the left side of each gel. Lane 1: adult globin chains from 129/Sv blood focus at pH 7·5 and 7·3. Lanes 2 and 3: embryonic globins from disrupted and washed visceral yolk sacs of 11-day 129/Sv embryos focus at pH 8·0, 7·7 and 7·1. Lane 4: embryonic globins found in disrupted and washed *in vitro* cystic embryoid bodies after 14 days in culture in bacterial dishes. The cells were cultured the first 10 days in standard medium and the last 4 days in 20 % foetal calf serum in Iscove's modified Dulbecco's medium. Benzidine reaction (lanes 1 and 2) and Coomassie blue staining (lanes 3 and 4). The cystic embryoid bodies were electrophoresed in their entirety in order to minimize the loss of erythrocytes. The visceral endodermal cells which greatly outnumber the erythrocytes in our samples are probably the source of the non-haemoglobin bands in lanes 2–4. Bar = 10 μm.

the myocard-like structures contained intercalated disks (Fig. 5C) which are heart and somitic myotome-specific intercellular junctions found where Z-bands of the apposing myofibrils of adjacent cells come together. The muscle cells could continue beating for more than a week. The morphological development of the *in vitro* beating structures was usually complete by the time the contractions were first observed.

## Visceral yolk-sac-derived blood islands

At the light microscopic level red areas could be detected just under the surface of approximately 1 % of the cystic embryoid bodies after 12 days of suspension culture (Fig. 6). Closer examination of similar blood islands found in cystic tumours using benzidine (Fig. 7A; stained erythrocyte cytoplasm being indicated by arrow) and fluorescence staining with TROMA 1 (Fig. 7B) revealed a pocket of blood cells surrounded by two endothelial layers. An electron micrograph (Fig. 8A) shows that the blood island erythrocytes of an *in vitro*-formed cystic embryoid body were nucleated — characteristic of the blood cells of embryonic visceral yolk sac. The haemoglobins of blood island cells in *in vitro*.cystic embryoid bodies were determined by isoelectric focusing to be embryonic (Fig. 8B, lane 4; control adult, lane 1; and control embryonic, lanes 2 (benzidine) and 3 (Coomassie blue) ). The double control shows that Coomassie-blue staining can also be used to detect haemoglobins in these structures. In cystic tumours the blood islands also contained exclusively embryonic haemoglobin (not shown), thus demonstrating that host erythrocytes had been excluded from the cystic tumours. As may be the case in the mouse embryo, the blood islands in the *in vitro* cystic embryoid bodies usually disappeared after 2–6 days.

In order to increase the frequency of appearance of blood islands, culture conditions used for blood stem cell cultures (Iscove's modified Eagle's medium) and for mouse embryo *in vitro* cultures (20 % human cord serum instead of foetal calf serum; Hsu, 1979) were combined. These culture conditions increased the percentage of cystic embryoid bodies which contained blood islands from 1 % to 30 % (Table 1). Four different cell lines from two different mouse strains gave similar results. These data suggest that except for possible small quantitative

Table 1. *Blood island production in various differentiated ES cell lines.*

| Mouse strain | Cell line | No. of exps. | Blood-island-containing cystic structures | |
| --- | --- | --- | --- | --- |
| | | | FCS | HCS |
| 129 | ES-D3/7 | 5 | 3/165 (2%)* | 78/249 (30%) |
| 129 | ES-D3/10/5 | 1 | 1/281 (1%) | 22/218 (19%) |
| 129 | ES-D3/4 | 1 | 0/102 (0%) | 19/76 (25%) |
| C57 | ES-632 | 2 | 3/34 (10%) | 16/48 (33%) |

ES cells were cultured in bacterial dishes in the absence of embryonic fibroblasts and in standard medium for 10 days. On day 10 the medium was changed to Iscove's modified Eagle's medium containing either 20% foetal calf serum (FCS) or human cord serum (HCS). Every 2 days thereafter each cystic, visceral yolk sac structure was examined under dark-field stereo optics. Each cystic structure containing one or more blood islands was scored as one. The scores from days 14–18 of culture were combined. Data were taken only from experiments in which both culture conditions were used.

* Number of blood-island-containing cystic embryoid bodies/total number examined.

differences between ES cell lines, they all display qualitatively similar developmental potentialities.

Immunofluorescence tests with a monoclonal antibody specific for macrophage cells (Fig. 9A and B) suggest that the cystic embryoid bodies may also contain
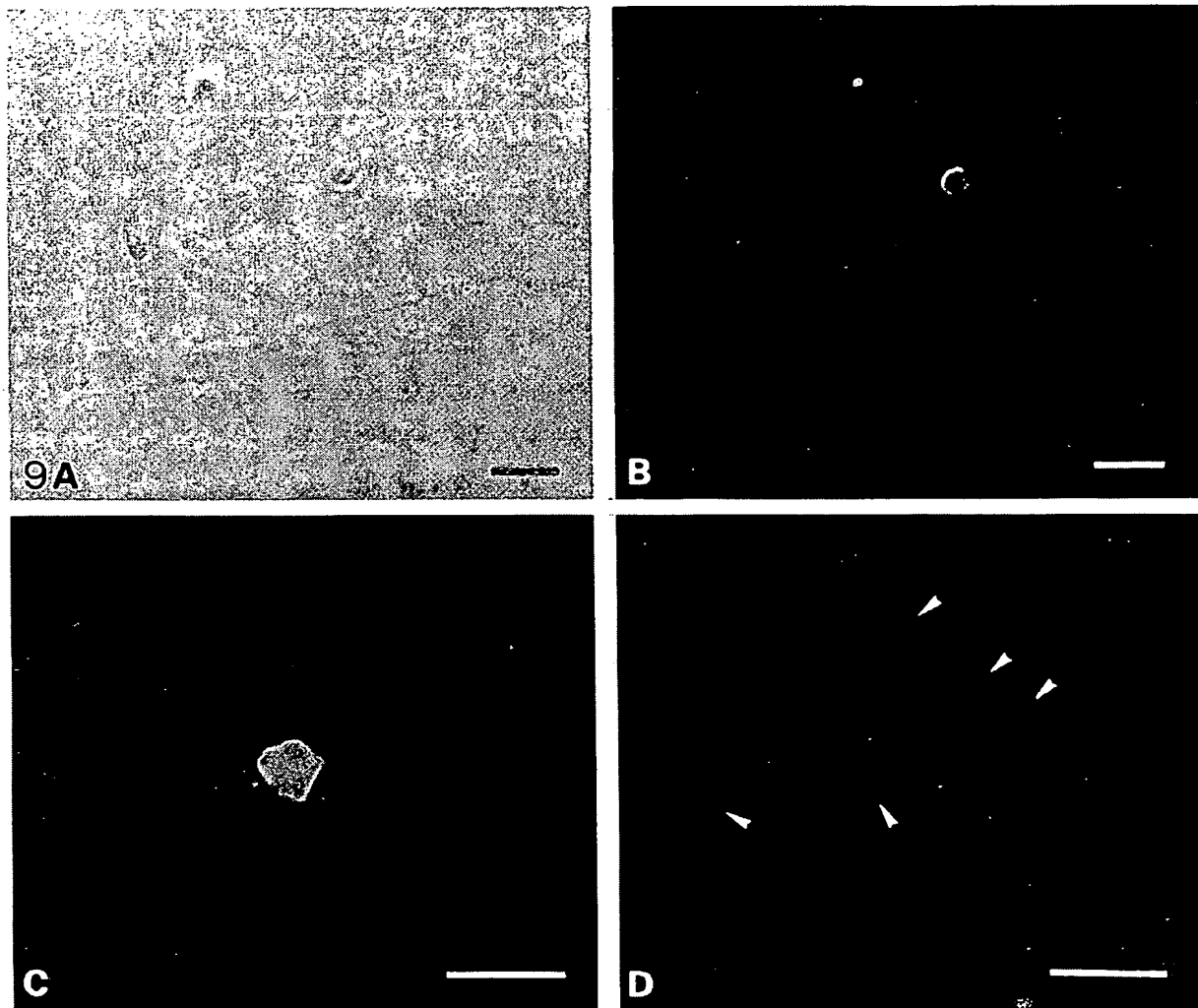


Fig. 9. Indirect immunofluorescence test with anti-macrophage monoclonal antibodies. (A,B) Positive control: Mouse peritoneal fluid was placed in tissue culture. After 2 days of culture, the attached cells were fixed and indirectly stained with antibody. Note the fibroblastic and non-fibroblastic cells which do not stain. (C,D) Cells were mechanically dissociated (see Materials and Methods section) from 18 day *in vitro* ES-D3 cystic embryoid bodies and cytocentrifuged onto microscope slides. (C) Indirect immunofluorescence test with antibody. (D) Control: Fluorescent second antibody alone. Arrowheads indicate location of cells. (A) Phase contrast. B–D, Fluorescence. Bars = 50 $\mu$m.

macrophages (Fig. 9C). Preliminary results using methyl cellulose culture conditions also indicate the presence of macrophage colony-forming cells in the cystic embryoid bodies (Gordon Keller, Basel Institute of Immunology, Switzerland).

## Comparison to EC cells

Two teratocarcinoma-derived cell lines have been reported to produce cystic structures which occasionally contained erythrocytes *in vitro* (Martin *et al.* 1977; Cudennec & Nicolas, 1977). We have compared a subclone (EC-PSA1/NG2) of one of them to our blastocyst-derived cell lines. The greatest difference between the ES and EC lines was of a quantitative nature (not shown). There appeared to be more mesoderm-derived structures in the ES cells, and although the teratocarcinoma-derived cells could form similar highly organized structures, the frequency with which they formed them was strikingly less.

## DISCUSSION

There are four aspects of blastocyst-derived embryonic stem cell lines which together make them potentially useful as a model system for embryonic development. The first is that they can be established from individual blastocysts of nearly any genotype. This makes it possible to investigate interstrain differences which may become apparent during embryogenesis. ES cell lines have been used for the investigation of cells homozygous for a lethal recessive mutation carried by inbred mouse strains (Magnuson *et al.* 1982), of gross chromosome abnormalities such as metacentricity (Kaufman, Robertson, Handyside & Evans, 1983), and of parthenogenesis (Robertson *et al.* 1983). Other uses could be to investigate the effects of mono- and trisomy (Gropp, 1982) and to investigate whether the differences between mouse strains which do and do not form spontaneous teratomas at high frequencies are caused by differences in the embryonic cells or in their host environment. Investigations of this nature could be of potential importance to the problem of transformation.

The second aspect, the consistency from one cell line to the next with respect to the *in vitro* developmental process, is necessary if the homozygous mutant lines referred to above are to be fruitfully compared to their respective background lines. We have now closely observed six ES cell lines from three different genetic backgrounds as well as many of their subclones (single cell as well as colony subclones) and have found them all to be remarkably similar with respect to their developmental characteristics.

In light of their developmental consistency, the third aspect of ES cell lines, namely their ease of establishment, is of special significance. One now has the advantage of always being able to work with cells which are temporally close to the embryo. It is generally accepted that the more time an embryonic cell spends outside of the embryonic environment, the more likely it will be selected for growth in the new environment. Consequently, both EC and ES cells can be expected to

lose their pluripotent characteristics over time. The special advantage of the blastocyst-derived ES cells is that under such circumstances a new line can easily be made with the same original potentialities of the old.

The fourth aspect of ES cells which make them suitable as a model system for embryonic development is their *in vitro* expression of a large degree of developmental potential. Since ES cells are capable of forming germ-line chimaeras at a frequency much higher than teratocarcinoma cells (Bradley *et al.* 1984), one would also expect higher *in vitro* frequencies of well-developed embryonic structures. This can be realized through two types of culture conditions: under two-dimensional (substrate-attached) culture conditions ES cells can differentiate into a large variety of cell types (Martin, 1981; this report). Such conditions may be well suited for investigations into some of the determining events which lead to terminal differentiation, especially if humoral factors are involved. In three-dimensional suspension culture ES cells form highly organized cystic embryoid body structures which are in many respects analogous to postimplantation embryos. With these structures one should be able to answer more easily questions concerning 'development' of the embryo rather than simply 'differentiation' of cell types. They may also be suitable for studying the developmental regulation of the expression of genes, normal or altered, inserted into ES cells, thereby offering all of the analytical advantages of *in vitro* systems. It is on these highly organized cystic embryoid bodies that we have focused our attention in this report.

## Comparison of ES cell development to that of the embryo and EC cells

The major proteins synthesized by the ES cell cystic embryoid bodies and secreted into their cavities are AFP and transferrin — two of the major products of the visceral yolk sac. It is noteworthy that other than these two proteins very few others are detectable. The minor proteins of approximately 25 000 and 45 000 $M_r$, but not the one of 300 000 $M_r$, have been observed previously in visceral yolk sac fluid (Adamson, 1982; Janzen, Andrews & Tamaoki, 1982). Recently, apolipoproteins of all three sizes have been found to be produced by visceral yolk sac endoderm (Shi & Heath, 1984; Meehan *et al.* 1984). We do not know why an approximately 300 000 $M_r$ protein is coprecipitated by anti-transferrin serum though not recognized by this same serum in immunoblots. This protein could not be coprecipitated from embryonic visceral yolk sacs metabolically labelled *in vitro* and is therefore unlikely to be apolipoprotein B.

In the embryo blood islands appear within the mesodermal layer of the visceral yolk sac on day 8. These primitive erythrocytes are large, contain nuclei and synthesize primitive haemoglobins (Craig & Russell, 1964). *In vitro* erythropoiesis has been shown to occur in two teratocarcinoma-derived EC cell lines. The large, nucleated red blood cells produced by the cystic structures of EC-PSA1 (Martin *et al.* 1977) and EC-PCC3/A/1 (organ culture conditions; Cudennec and Nicolas, 1977) develop blood islands from mesodermal thickenings on the inner side of

endodermal vesicles. The EC-PCC3/A/1 blood cells contain embryonic haemoglobin (Cudennec, Thiery & Le Douarin, 1979).

Under standard culture conditions approximately 1 % of the blastocyst-derived ES cell cystic bodies contain islands of large, nucleated, embryonic haemoglobin-containing erythrocytes after two weeks in culture. A 30-fold increase in the percentage of blood island-containing cystic bodies can be induced by human cord serum. That these cells could make adult haemoglobin under appropriate conditions, however, cannot be ruled out. This is important in light of evidence that some sera used in culture (Stamatoyannopoulos, Nakamoto, Kurachi & Papayannopoulou, 1983) as well as other embryonic tissue (Cudennec et al. 1981; Ripoche & Cudennec, 1983; Labastie, Thiery & Le Douarin, 1984) can induce yolk sac erythrocytes to produce adult haemoglobin. The cystic embryoid bodies may also contain stem cells for macrophages. The existence of other stem cells of the haemopoietic lineage is presently under investigation. The presence of haemopoietic stem cells in the cystic structures may provide investigators with a purely in vitro model system for unravelling some of the complexities of the haemopoietic cell lineages.

In the embryo the first muscle cells to appear are in the myocardium and the somitic myotome. Whereas the myotome-derived muscle anlagen produce multi-nucleated cells, the myocardial cells remain by and large mononucleated and develop intercalated disks which serve to join the myofibrillar apparatus of adjoining cells (rev. by Manasek, 1973). Contractile protein isoforms also seem to follow this pattern in that cardiac isoforms are found both in myotome and myocard, but not in skeletal muscle (Toyota & Shimada, 1981; Sweeney et al. 1984). The only distinguishing characteristic then between the earliest myocardial and myotomal muscle is the rhythmic contraction of the primitive heart cells.

The production of beating muscle cells by two teratocarcinoma-derived cell lines has been reported. EC-PSA1 cells produce such cells both in monolayer culture (Martin et al. 1977) and in cystic embryoid bodies (our observations). EC-P19 cells, which require chemical inducers to differentiate, form beating structures in monolayer culture (McBurney et al. 1982). The development of associated endocardial tissue by these cell lines has not been described previously.

About one third of the cystic embryoid bodies produced by the blastocyst-derived cells develop rhythmically contracting, intercalated disk-containing myocardial cells. The associated endocardial tissue found in cultures of substrate-attached cells can also form in the cystic structures (not shown). These data show that ES cells have the potential to develop into several cardiac cell types in a well-organized manner, suggesting that they may be suitable for investigations of heart organogenesis.

It is pertinent to this discussion that only the embryonic and not the extra-embryonic portion of egg-cylinder-stage embryos can 1) form teratocarcinomas (Diwan & Stevens, 1976) or 2) develop in vitro into structures similar to those described here (Hogan & Tilly, 1981). Likewise, it is noteworthy that the structures

described in this report are similar to those produced by some of the earlier attempts at embryo culture (Hsu, 1972). These data are consistent with the chimaera experiments mentioned above (Bradley *et al.*, 1984) and lead to the conclusion that ES cells are in fact quite similar to the pluripotent cells of the blastocyst. We are confident that the developmental similarity of most of the ES cell lines produced, their ease of establishment, their ability to form highly organized structures analogous to those of the embryo, and their amenability to the production of interstrain variants, should provide investigators with new approaches to the study of embryonic development.

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